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IMMUNOMODULATORY APPROACHES AFTER EXPERIMENTAL ISCHEMIC STROKE

Jenni Anttila

ACADEMIC DISSERTATION

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ABSTRACT

Ischemic stroke is one of the leading causes of death and disability worldwide but the treatment options remain limited. Ischemic stroke, or cerebral infarct, occurs when blood flow to a focal brain region is restricted due to arterial blockage. Lack of oxygen and energy leads to rapid neuronal death in the ischemic region and to an inflammatory response *via* activation of brain-resident immune cells, microglia, and infiltration of peripheral leukocytes after ischemia-induced blood-brain barrier damage. Acute neuroprotective strategies need to be executed within a few hours after ischemia induction to be effective and have not proven successful in clinical trials. However, inflammation persists in the post-stroke brain and modulation of post-stroke inflammation could provide a therapeutic strategy with a large time window. Inflammation has both beneficial and harmful effects on injury progression but our understanding of many aspects of post-stroke inflammation remains incomplete.

We characterized the neuroinflammatory response in the rat distal middle cerebral artery occlusion (dMCAo) model that was used to induce cortical infarcts in this thesis work. We found long-lasting inflammation and presence of phagocytic cells for up to 4 months after dMCAo, especially in the ipsilateral thalamus. We also found delayed neuronal loss occurring in the ipsilateral thalamus between 1-2 weeks after dMCAo due to connecting projection pathways between the cortex and the thalamus.

Mesencephalic astrocyte-derived neurotrophic factor (MANF) is an 18 kDa endoplasmic reticulum luminal protein that is neuroprotective in experimental ischemic stroke models and has been associated with immunomodulatory properties. However, the knowledge of MANF's recovery-promoting effects, mechanism of action, and endogenous expression pattern after cerebral ischemia are still limited. Thus, we characterized the endogenous MANF protein expression pattern in the dMCAo model and in ischemic stroke patient brains. Notably, we found that MANF protein expression is strongly induced in activated immune cells in the infarcted rodent and human brains. We also studied intracerebral post-stroke MANF therapy *via* viral delivery and recombinant protein injection and found that MANF promotes functional recovery when administered into the brain 2 days post-stroke as an adeno-associated viral (AAV) vector or as a recombinant protein starting 3-7 days post-stroke. Post-stroke MANF treatment did not alter the infarct size but the AAV-MANF therapy induced a transient increase in the number of phagocytic cells and innate immunity-related transcript levels in the peri-infarct area. In addition, we conducted a proof-of-concept study using intranasal MANF delivery to explore alternative delivery routes for

administering the blood-brain barrier impermeable MANF protein. Pre-stroke intranasal MANF therapy decreased infarct volume and behavioral deficits. These data suggest a theoretical potential for intranasal MANF therapy, but bioavailability requires further improvement.

As another approach, we investigated the efficacy of repeated post-stroke intranasal (+)-naloxone delivery in the dMCAo model. (-)-Naloxone is a small molecule drug which has been in clinical use for opioid overdose for decades and studied in the acute treatment of ischemic stroke because of its opioid receptor antagonizing effect. More recently, (-)-naloxone and its opioid receptor inactive (+) enantiomer have been shown to possess anti-inflammatory effects and to reduce microglial activation. (+)-Naloxone therapy, started one day post-stroke and continued for 7 days, decreased the infarct size and microglia/macrophage activation, and reduced behavioral deficits.

This work broadens knowledge of the post-stroke neuroinflammation and secondary pathology of the thalamus in the cortical infarct model and shows for the first time that endogenous MANF protein is expressed in the activated, phagocytic immune cells in the infarcted human brain. This work also provides evidence on the recovery-promoting effects of post-stroke MANF and (+)-naloxone therapy and links both therapies with immunomodulatory functions.

TIIVISTELMÄ

Aivoinfarkti on maailmanlaajuisesti yksi yleisimmistä kuolinsyistä sekä toimintakykyä heikentävistä tekijöistä, mutta sen hoitokeinot ovat rajalliset. Aivoinfarkti aiheutuu valtimotukoksesta, joka estää veren virtauksen paikalliselle aivoalueelle. Hapen ja energian puute johtaa nopeasti hermosolujen kuolemaan aivoalueella sekä tulehdukselliseen vasteeseen aivojen paikallisten immuunisolujen, mikroglian, aktivoiduttua sekä perifeeristen valkosolujen tunkeuduttua aivokudokseen rikkoutuneen veri-aivoesteen läpi. Akuutit solukuoleman vähentämiseen tähtäävät hoidot täytyy toteuttaa muutaman tunnin kuluessa infarktista. Aivoinfarktiin liittyvä inflammaatio on kuitenkin pitkäkestoinen ja tämän tulehdusvasteen muokkaaminen voisi tarjota hoitokeinon, jolla on laaja aikaikkuna. Inflammaatiolla on sekä myönteisiä että haitallisia vaikutuksia vaurion etenemisen kannalta, mutta ymmärryksemme näistä on monelta osin vielä puutteellista.

Karakterisoimme aivojen inflammatorisen vasteen rotan distaalisen keskimmäisen aivovaltimon okkluusio-mallissa, jota käytettiin aivokuoren infarktin aiheuttamiseen tässä väitöstyössä. Havaitsimme pitkäkestoista inflammaatiota ja fagosytoivia soluja aivoissa vielä 4 kuukauden päästä infarktista erityisesti vaurion puoleisella talamuksen aivoalueella. Havaitsimme myös viivästynyttä hermosolukuolemaa vaurion puoleisessa talamuksessa 1-2 viikon kuluessa infarktista johtuen aivokuoren ja talamuksen yhdistävistä hermoradoista.

Keskiaivojen astrosyyttiperäinen hermokasvutekijä (MANF) on 18 kDa:n kokoinen solulimakalvoston sisäinen proteiini, joka suojaa vaurioilta aivoinfarktin kokeellisissa malleissa ja jolla on immuunivastetta säätteleviä ominaisuuksia. Tiedot MANF:n toipumista edistävistä vaikutuksista, vaikutusmekanismista sekä ilmentymisestä aivoinfarktin jälkeen ovat kuitenkin vielä puutteelliset. Karakterisoimme MANF-proteiinin ilmentymisen aivokuoren infarktimallissa sekä aivoinfarktipotilaiden aivonäytteissä ja havaitsimme MANF:n ilmentymisen lisääntyvän voimakkaasti aktivoituneissa immuunisoluissa sekä jyrsijöiden että potilaiden aivoissa infarktin jälkeen. Tutkimme myös infarktin jälkeen aivoinjektiona annetun MANF-hoidon vaikutusta rottien toipumiseen ja havaitsimme virusvektorina 2 päivää infarktin jälkeen annetun MANF:n sekä proteiinina 3-7 päivää infarktin jälkeen aloitetun MANF-annostelun edistävän toiminnallista toipumista. Infarktin jälkeinen MANF-hoito ei vaikuttanut infarktin kokoon, mutta virusvektorina annettu MANF lisäsi tilapäisesti fagosytoivien solujen määrää sekä immuunivasteeseen liittyvien geenien ilmentymistä infarktia ympäröivällä alueella. Lisäksi suoritimme rotilla

alustavan tutkimuksen käyttämällä intranasaalista MANF-annostelua selvittääksemme vaihtoehtoisia tapoja veri-aivoesteen läpäisemättömän MANF-proteiinin annostelemiseksi. Ennen infarktia intranasaalisesti annettu MANF pienensi infarktin kokoa sekä vähensi toiminnallisia vajeita. Tämä viittaa intranasaalisen MANF-hoidon teoreettiseen toimivuuteen, mutta MANF:n biologinen hyötyosuus vaatii parantamista.

Toisena lähestymistapana tutkimme infarktin jälkeisen toistetun intranasaalisen (+)-naloksonin annostelua aivokuoren infarktimallissa. (-)-Naloksoni on opioidiyliannostuksen hoitoon jo kauan kliinisessä käytössä ollut pienimolekyylinen yhdiste, jota on tutkittu aivoinfarktin akuutissa hoidossa opioidireseptoreja salpaavan vaikutuksensa takia. (-)-Naloksonin sekä sen opioidireseptoreihin sitoutumattoman (+)-enantiomeerin on hiljattain todettu omaavan anti-inflammatorisia vaikutuksia sekä vähentävän mikroglian aktivaatiota. Havaitsimmekin päivä infarktin jälkeen aloitetun viikon kestävän (+)-naloksoni-hoidon pienentävän infarktin kokoa sekä vähentävän mikroglian ja makrofagien aktivaatiota sekä rottien toiminnallisia vajeita.

Tämä väitöstyö laajentaa tietämystämme aivoinfarktin jälkeisestä tulehduksellisesta vasteesta sekä talamuksen sekundäärisestä vauriosta aivokuoren infarktimallissa ja osoittaa ensimmäisen kerran MANF-proteiinin ilmentyvän aktivoituneissa, fagosytoivissa immuunisoluissa aivoinfarktipotilaiden aivoissa. Väitöstyö todistaa myös infarktin jälkeen annetun MANF- ja (+)-naloksoni-hoidon edistävän rottien toipumista sekä liittää molemmat hoidot immuunivastetta säätteleviin ominaisuuksiin.

CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications:

I **Anttila JE**, Albert K, Wires ES, Mätlik K, Loram LC, Watkins LR, Rice KC, Wang Y, Harvey BK, Airavaara M: Post-stroke intranasal (+)-naloxone delivery reduces microglial activation and improves behavioral recovery from ischemic injury. *eNeuro* 5(2), 2018. pii: ENEURO.0395-17.2018

II Mätlik K, **Anttila JE**,* Tseng KY,* Smolander OP, Pakarinen E, Lehtonen L, Abo-Ramadan U, Lindholm P, Zheng C, Harvey B, Arumäe U, Lindahl M, Airavaara M: Poststroke delivery of MANF promotes functional recovery in rats. *Science Advances* 4(5): eaap8957, 2018

III **Anttila JE**, Mattila OS, Tseng KY, Mätlik K, Lindholm P, Lindahl M, Lindsberg PJ, Airavaara M: MANF protein expression in brain myeloid cells after ischemic stroke: study translating from rodent to human. *Manuscript*.

IV **Anttila JE**, Pöyhönen S, Airavaara M: Secondary pathology of the thalamus after focal cortical stroke in rats is not associated with thermal or mechanical hypersensitivity and is not alleviated by intra-thalamic post-stroke delivery of recombinant CDNF or MANF. *Cell Transplantation 28(4): 425-438*, 2019

* equal contribution

The publications are referred to in the text by their roman numerals. Reprints were made with the permission of the copyright holders.

Contributions for the publications:

I

Study design: JEA, LRW, YW, BKH, MA
Laboratory work and data analysis
- Animal work for model characterization: JEA, MA
- Animal work for naloxone study: ESW, BKH, MA
- Immunohistochemistry: JEA, KAA
- CD11b+ cell isolation and TNF-α ELISA: JEA, KM
- (+)-naloxone synthesis: KCR
- LCL, LRW: qPCR (data not included in the final article)

Manuscript writing
- Original draft: JEA
- Review and editing: KA, ESW, KM, LCL, LRW, KCR, YW, BKH, MA

II

Study design: KM, JEA, TKY, OPS, UA, MA
Laboratory work and data analysis
- Animal work: KM, JEA, TKY, MA
- MRI: UAR, KM
- Immunohistochemistry: KM, JEA, CZ
- qPCR: KM, EP
- RNA sequencing: OPS, LL, KM

Manuscript writing
- Original draft: KM
- Review and editing: JEA, MA

III

Study design: JEA, OSM, PJL, MA
Laboratory work and data analysis
- Animal work: JEA, TKY, MA
- Iodination of MANF: KM
- Immunohistochemistry: JEA
- ELISA: JEA, PL

Manuscript writing
- Original draft: JEA
- Review and editing: OSM, PL, ML, PJL, MA

IV

Study design: JEA, MA
Laboratory work and data analysis
- Animal work: JEA, SP, MA
- Immunohistochemistry: JEA, SP

Manuscript writing
- Original draft: JEA
- Review and editing: SP, MA

ABBREVIATIONS

6-OHDA	6-hydroxydopamine
AAV	adeno-associated virus
Arg1	arginase 1
ARP	arginine-rich protein
ARMET	arginine-rich, mutated in early stage of tumors
ATF6	activating transcription factor 6
Akt	protein kinase B
ATP	adenosine triphosphate
BBB	blood-brain barrier
BDNF	brain-derived neurotrophic factor
C	carboxy
C3	complement component 3
CBF	cerebral blood flow
CCL2	C-C motif chemokine ligand 2
CCR2	C-C chemokine receptor 2
CD	cluster of differentiation
cDNA	complementary deoxyribonucleic acid
CDNF	cerebral dopamine neurotrophic factor
CHOP	C/EBP homologous protein
CNS	central nervous system
CPM	counts per minute
CRP	C-reactive protein
CX3CR1	CX3C chemokine receptor 1
CXXC	two cysteines separated by two other residues
dMCAo	distal middle cerebral artery occlusion
DmMANF	*Drosophila melanogaster* MANF
DNA	deoxyribonucleic acid
ELISA	enzyme-linked immunosorbent assay
Emr1	EGF module-containing mucin-like receptor 1
ER	endoplasmic reticulum
GDNF	glial cell line-derived neurotrophic factor
GFAP	glial fibrillary acidic protein
GFP	green fluorescent protein
$gp91^{phox}$	91 kDa glycoprotein
GRP78	78 kDa glucose-regulated protein
Iba1	ionized calcium-binding adapter molecule 1
IL	interleukin
IGF-1	insulin-like growth factor 1
IRE1	inositol-requiring enzyme 1
IRF3	interferon regulatory factor 3
KDEL	canonical ER retention signal

ko	knockout
LPS	lipopolysaccharide
MANF	mesencephalic astrocyte-derived neurotrophic factor
Manf-1	*Caenorhabditis elegans* MANF
MBP	myelin basic protein
MCAo	middle cerebral artery occlusion
MCP-1	monocyte chemoattractant protein 1
MDM2	murine double minute 2
mRNA	messenger ribonucleic acid
MRC1	mannose receptor C-type 1
mTor	mechanistic target of rapamycin
N	amino
NADPH	nicotinamide adenine dinucleotide phosphate
NeuN	neuronal nuclei
NGF	nerve growth factor
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NO	nitric oxide
NOX2	NADPH oxidase 2
p53	tumor suppressor p53
PERK	double-stranded RNA-activated protein kinase-like ER kinase
PHOX	phagocyte oxidase
PI3K	phosphatidylinositol-3 kinase
rhMANF	recombinant human MANF
RNA	ribonucleic acid
ROS	reactive oxygen species
RTDL	KDEL-like sequence
rtPA	recombinant tissue plasminogen activator
S100A8	calgranulin A
S100A9	calgranulin B
SCG	superior cervical ganglion
SDMANF	*Suberites domuncula* MANF
SEM	standard error of the mean
SRRR	Stroke Recovery and Rehabilitation Roundtable
STAIR	Stroke Therapy Academic Industry Roundtable
STAT3	signal transducer and activator of transcription 3
SVZ	subventricular zone
TBI	traumatic brain injury
TGF-β	transforming growth factor β
TLR4	Toll-like receptor 4
TNF-α	tumor necrosis factor α
TTC	2,3,5-triphenyl-2H-tetrazolium chloride
UPR	unfolded protein response
VEGF	vascular endothelial growth factor

1 INTRODUCTION

Stroke is the second leading cause of death and a major cause of disability worldwide, making the economic burden of stroke-related costs enormous. Age is the most important risk factor for stroke and with the aging population, the incidence of stroke is expected to further increase. Yet, treatment options are limited and there is no drug treatment that would promote functional recovery after stroke. Currently, the only pharmacological therapy for stroke in the US and Europe is the thrombolytic agent alteplase that needs to be administered routinely within 4.5 hours from the onset of ischemic stroke. This narrow time window can be accomplished in only a minority of patients. Thus, it is vital to develop a drug therapy that would aid recovery and could be administered even days after the initial ischemic attack.

Approximately 80% of stroke cases are caused by ischemia which usually results from local thrombosis or an embolus blocking a major cerebral artery. Oxygen and energy depletion leads rapidly to neuronal death in the ischemic core area. Also, the immune cells of the brain, microglia, are activated and peripheral immune cells infiltrate the ischemic area after blood-brain barrier (BBB) damage. Inflammation is one of the key players in the pathogenesis of ischemic stroke but is also an essential element in brain repair mechanisms, and modulation of post-stroke inflammation as a therapeutic target has gained an increasing amount of attention during the past years. Post-stroke inflammation is long-lasting and could thus provide a wide therapeutic time window.

Mesencephalic astrocyte-derived neurotrophic factor (MANF) is a cytoprotective protein residing in the endoplasmic reticulum (ER) lumen and is an important regulator of ER homeostasis (Lindahl *et al.*, 2017). MANF is neuroprotective in the rat middle cerebral artery occlusion model when given before stroke as a protein or *via* a viral vector (Airavaara *et al.*, 2009; Airavaara *et al.*, 2010). Interestingly, MANF has been shown to possess immunomodulatory effects (Chen *et al.*, 2015; Neves *et al.*, 2016).

Naloxone is an old drug with relatively newly found anti-inflammatory properties (Das *et al.*, 1995; Liu *et al.*, 2000c; Hutchinson *et al.*, 2008). The (-) enantiomer of naloxone is a potent opioid receptor antagonist in clinical use for opioid overdose (Iijima *et al.*, 1978). Naloxone has good brain penetration properties and has become one of the most used drugs for opioid overdose, and is nowadays given most often intranasally due to ease of administration (Rzasa Lynn & Galinkin, 2018). However, the (+) enantiomer has a very low affinity for opioid receptors (Iijima *et al.*, 1978) but possesses similar anti-inflammatory effects as (-)-naloxone, making (+)-naloxone an interesting candidate with more specificity towards inflammation.

As deeper knowledge of post-stroke inflammation and novel therapies for ischemic stroke are desperately needed, this thesis focused on investigating post-stroke inflammation and the effects of post-stroke MANF and (+)-naloxone therapy.

2 REVIEW OF THE LITERATURE

2.1 ISCHEMIC STROKE

Stroke encompasses two major types of stroke: ischemic stroke and hemorrhagic stroke. Cerebral ischemic stroke is caused by local thrombosis or embolism that leads to a lack of blood supply to a focal area in the brain, whereas hemorrhagic stroke is caused by intracerebral or subarachnoid hemorrhage (Hankey, 2017). The majority, about 80%, of strokes are caused by ischemia (Meretoja *et al.*, 2010).

The term "stroke" was first introduced by a physician named William Cole in 1689 (Cole, 1689) but it took more than 200 years to become an established term in medicine. The term "apoplexy", derived from ancient Greek and meaning "to strike suddenly", was used until the 20th century to describe stroke (Engelhardt, 2017). The first written description of apoplexy was documented by Hippocrates approximately 2,500 years ago (Engelhardt, 2017). Hippocrates' definition of stroke still stands: "It is impossible to remove a strong attack of apoplexy, and not easy to remove a weak attack" (Marks, 1818), as modern medicine is still challenged by limited treatment options for stroke.

Globally, and in Finland, stroke is the second leading cause of death after ischemic heart disease (Mortality & Causes of Death, 2016). In Finland, there are approximately 18 000 new cases of ischemic stroke every year (Aivoliitto, 2020). The costs of stroke treatment are huge, and in Finland encompassed 3% of the entire national healthcare expenditure in 2017 with costs of 640 million euros total related to stroke (Luengo-Fernandez *et al.*, 2019). Within 1 year post-stroke, the fatality of ischemic stroke is 24%, while 64% of the patients are able to live at home, and 12% remain in institutional care (Meretoja *et al.*, 2010). The incidence of ischemic stroke increases with age (Seshadri *et al.*, 2006). Other risk factors for ischemic stroke are hypertension, dyslipidemia, carotid stenosis, atrial fibrillation, diabetes, cigarette smoking, excessive alcohol consumption, drug use, obesity, unhealthy diet, physical inactivity, depression, and psychosocial stress (Autret *et al.*, 1987; Lewington *et al.*, 2002; O'Donnell *et al.*, 2010; Fonseca & Ferro, 2013). The neurological symptoms of focal ischemic stroke are dependent on the brain region that is affected by the ischemia and have a sudden onset. The symptoms typically include unilateral weakness or numbness of arm or leg, facial weakness, speech disturbances, visual field defects or visual loss, and vertigo (Hankey, 2017).

Rapid recanalization is the foundation of ischemic stroke therapy. The importance of rapid thrombolysis was depicted well in a study that found every 15 min decrease in treatment delay provided 1 month of extra disability-free life (Meretoja *et al.*, 2014). Currently, the only drug approved for clinical use in Europe and the US is the recombinant tissue plasminogen activator (rtPA) alteplase that is used for thrombolysis and in general needs to be administered within 4.5h from the symptom onset (Hacke *et al.*, 2008). According to recent studies, thrombolysis is beneficial in some patients for up to 9h after the symptom onset (Thomalla *et al.*, 2018; Campbell *et al.*, 2019). However, due to a narrow time window and contraindications, only a minority of all ischemic stroke patients receive thrombolysis, leaving the majority of the patients without the possibility for drug treatment. Finland is one of the top countries in thrombolysis rates with 15% of ischemic stroke patients receiving thrombolysis (Stevens *et al.*, 2017) and with "door-to-needle time" of 26 min on average (Meretoja *et al.*, 2014). A mechanical procedure for recanalization, endovascular thrombectomy, can be performed at least until 6h after the symptom onset (Goyal *et al.*, 2016; Hankey, 2017) and in some patients up to 24h (Albers *et al.*, 2018; Nogueira *et al.*, 2018). Hemicraniectomy can be performed in patients with large, malignant middle cerebral artery infarctions to reduce intracranial hypertension caused by cerebral swelling (Yang *et al.*, 2015). In this patient group, the hemicraniectomy decreases mortality and can increase functional outcome (Yang *et al.*, 2015). After the ischemic stroke, current therapies aim to prevent the renewal of infarction primarily with antithrombotic agents and treatment of known risk factors, such as hypertension, dyslipidemia, and obesity (Hankey, 2017). Rehabilitative therapy is based on physiotherapy and neuropsychological rehabilitation. However, recovery is often slow and incomplete and there is a great need for a drug therapy that could promote the functional recovery of the stroke survivors.

2.1.1 PATHOPHYSIOLOGY OF ISCHEMIC STROKE

Focal ischemic stroke is caused by hypoperfusion in the brain territory supplied by the occluded artery, and the extent of cerebral injury depends on the severity and duration of hypoperfusion [see in (Dirnagl *et al.*, 1999; Durukan & Tatlisumak, 2007; Brouns & De Deyn, 2009)]. The infarct core is dependent on the supply of the occluded artery, and the neuronal cells are rapidly and permanently lost due to ischemia. It is estimated that each minute during ischemia, 1.9 billion neurons are lost (Saver, 2006). The core is surrounded by an ischemic penumbra, where the collateral vessels are able to compensate for the hypoperfusion to some extent. However, if the hypoperfusion continues, the infarct core also expands to the penumbra area

(Baron, 1999). The main mechanism of cell death in the infarct core is necrosis, while apoptosis dominates in the penumbra (Sairanen *et al.*, 2006).

The focal cerebral hypoperfusion causes oxygen and energy depletion in the surrounding cerebral tissue and triggers the "ischemic cascade" (Figure 1). Cerebral tissue is extremely dependent on the continuous blood supply as the brain has no energy store (Durukan & Tatlisumak, 2007). The ischemic cascade initiates from adenosine triphosphate (ATP) depletion that leads to failure of the ATP-dependent Na^+/K^+- and Ca^{2+}/H-ion pumps and subsequent depolarization of cells. The depolarization activates voltage-gated Ca^{2+} channels and results in the release of excitatory amino acids, especially glutamate, to the extracellular space. By activating glutamate receptors, glutamate induces Ca^{2+} influx into the cell and further exacerbates the increase of intracellular Ca^{2+} levels. Excess extracellular glutamate and K^+ also trigger depolarization in the surrounding penumbra area which leads to permanent damage if long-lasting (Dijkhuizen *et al.*, 1999).

Simultaneously, elevated intracellular Ca^{2+} concentration induces activation of several Ca^{2+} dependent enzymes (ex. kinases, proteases, lipases, and synthases) and production of cytotoxic compounds, such as reactive oxygen species (ROS) and nitric oxide (NO). The free radicals damage various cellular structures and can trigger apoptosis, and the activation of proteases and lipases leads to degradation of the plasma membrane and necrosis. Moreover, the activation of matrix metalloproteinases and damage to vascular endothelium leads to disruption of the BBB and subsequent vasogenic edema as well as leukocyte infiltration. In an animal model of transient ischemic stroke, the BBB already opens 25 min after reperfusion and remains open up to 5 weeks post-stroke (Strbian *et al.*, 2008; Abo-Ramadan *et al.*, 2009). The level of BBB disruption is dependent on the infarct size (Abo-Ramadan *et al.*, 2009). Reperfusion is the aim of ischemic stroke therapy but can paradoxically exacerbate the cerebral injury *via* increased ROS formation in ischemia-compromised mitochondria combined with inadequate antioxidant production during ischemia, subsequent activation of the pro-inflammatory transcription factor NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) in the vascular endothelium, and enhanced neutrophil adhesion [see in (Schaller & Graf, 2004)]. Reperfusion can also induce lipid peroxidation and membrane damage, and surprisingly, increase apoptosis which is an energy-dependent process.

Increased intracellular Ca^{2+} levels and ATP deficiency disrupt the ER Ca^{2+} homeostasis by inducing Ca^{2+} release from the ER *via* ryanodine and inositol trisphosphate receptor channels and *via* failure of the ATP-dependent sarcoplasmic/endoplasmic Ca^{2+}-ATPase pump that maintains the high Ca^{2+} levels within the ER under physiological conditions [see in (Bodalia *et al.*, 2013)]. Disruption of ER Ca^{2+} homeostasis leads to the accumulation of unfolded proteins in the ER lumen and triggers ER stress and the unfolded

protein response (UPR). In general, the adaptive responses of the UPR aim to restore ER homeostasis but, when failing, apoptosis can be induced [see in (Hetz, 2012)]. However, the role of ER Ca^{2+} depletion and UPR in the modulation of cell death in ischemic stroke is not well known.

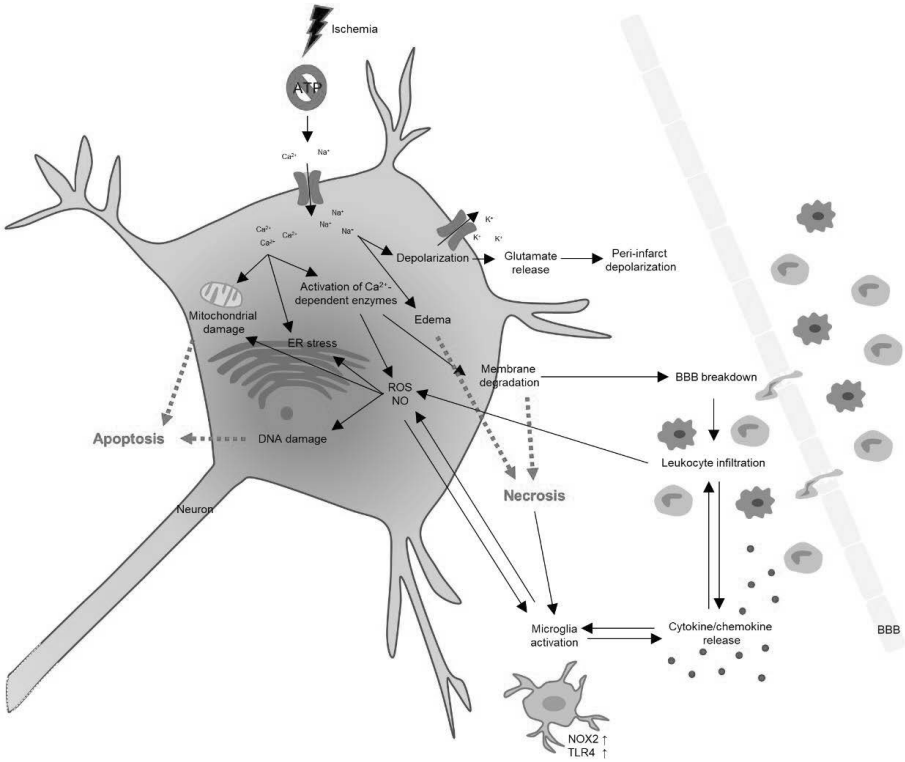

Figure 1. Simplified schematic image of pathophysiological events occurring after focal cerebral ischemia in the infarct core. Ischemia causes a lack of oxygen and energy (ATP) which leads to ion pump failure and increased intracellular Na^+ and Ca^{2+} levels and extracellular K^+ levels. Elevated Ca^{2+} results in mitochondrial damage, ER stress, activation of enzymes that produce ROS and NO causing further damage to different cellular organs and degradation of cellular membranes. Elevated Na^+ results in cellular edema and the cell membrane is depolarized after ion pump failure, leading to glutamate release and further depolarization of peri-infarct region cells as well. The BBB is enzymatically degraded and allows the infiltration of peripheral leukocytes into the brain parenchyma. Also, resident microglia are activated and further exacerbate the inflammatory cycle. NOX2 and TLR4 levels are increased in the activated microglia and contribute to ROS and cytokine production.
ATP, adenosine triphosphate; BBB, blood-brain barrier; ER, endoplasmic reticulum; NO, nitric oxide; NOX2, NADPH oxidase 2; ROS, reactive oxygen species; TLR4, Toll-like receptor 4

2.1.2 BRAIN REPAIR MECHANISMS AFTER ISCHEMIC STROKE

Ischemic stroke is associated with significant spontaneous recovery that occurs mainly within the first 2 months post-stroke and may continue later in patients with severe deficits (Nakayama *et al.*, 1994). Intrinsic brain repair mechanisms are initiated soon after ischemic injury, already within 1-2 days in rodents (Zhang *et al.*, 2001; Hayashi *et al.*, 2003) and 5 days in patients, continuing for months or more (Krupinski *et al.*, 1994). These processes are orchestrated together by different cell types in the brain to create a repair-promoting environment and consist of angiogenesis, neurogenesis, synaptogenesis and white matter remodeling, and neuroinflammation [see in (Venkat *et al.*, 2018)]. In a wider sense, this intrinsic ability of the brain to remodel for optimized function can be considered plasticity and functional recovery occurs when other brain regions adjacent to the ischemic lesion and in the contralesional hemisphere take functions of the damaged neuronal networks [see in (Murphy & Corbett, 2009; Guggisberg *et al.*, 2019)]. The timing of these reparative processes and different therapeutic approaches after ischemic stroke are depicted in Figure 2. The biological processes in the stroke recovery phase differ substantially from the processes in the hyperacute and acute phase targeted by neuroprotective treatments, and these differences should be considered when conducting stroke recovery trials (Corbett *et al.*, 2017).

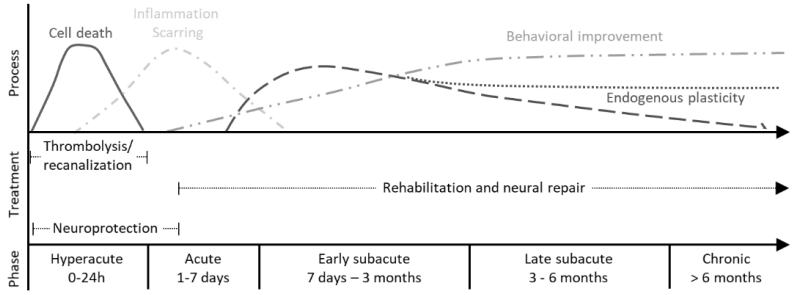

Figure 2. Timeline of pathophysiological and reparative processes and therapeutic approaches after ischemic stroke. The goal of reparative treatments is to enhance endogenous plasticity (shown in pointed purple line). Adapted from (Dobkin & Carmichael, 2016; Bernhardt *et al.*, 2017).

Angiogenesis, meaning the formation of new microvessels, is a highly regulated phenomenon and the gene expression of angiogenic factors is already induced 1h after cerebral ischemia in the middle cerebral artery occlusion (MCAo) model in mice (Hayashi *et al.*, 2003). In ischemic stroke patients, angiogenesis has been shown to occur in the penumbra and linked with better survival (Krupinski *et al.*, 1994).

Neurogenesis occurs in the post-stroke brain in the rodent MCAo model (Arvidsson *et al.*, 2002) and has been reported in the immunostained

penumbra area of the infarcted human cortex as well (Jin *et al.*, 2006). Using a photothrombotic stroke model in mice, neurogenesis was recently shown to be necessary for post-stroke functional recovery (Liang *et al.*, 2019). However, a study using radiocarbon determination found no neurogenesis in the infarcted human cortex, making the role of endogenous neurogenesis in the human post-stroke brain unclear (Huttner *et al.*, 2014).

In an ischemic stroke model, markers of axonal growth have been reported to increase in the peri-infarct cortex already 3 days after distal middle cerebral artery occlusion (dMCAo) in rats and synaptogenesis to occur at 14 days post-dMCAo and onwards in the peri-infarct cortex as well as in the contralateral cortex (Stroemer *et al.*, 1995). Formation of new synapses and white matter remodeling, including remodeling of axonal connections and oligodendrogenesis-induced remyelination, are important for restoring neuronal connections in the damaged brain after ischemic stroke and have been linked to behavioral recovery.

Neuroinflammation has a role in creating a pro-regenerative environment after ischemic injury, and glial cells, including astrocytes and microglia, support the repair mechanisms by producing trophic factors, such as brain-derived neurotrophic factor (BDNF), glial cell line-derived neurotrophic factor (GDNF) and vascular endothelial growth factor (VEGF) [see in (Hermann & Chopp, 2012)].

Boosting the endogenous repair mechanisms has created one line of research for stroke therapy. The time window of potential neuroreparative therapies is rather large, and thus appealing from a clinical point of view. The main therapeutic lines investigated are based on exogenous cell transplantation, stimulation of endogenous neurogenesis, and neuronal reprogramming.

2.2 INFLAMMATION AFTER ISCHEMIC STROKE

Ischemic stroke induces activation of brain-resident immune cells, microglia, and infiltration of peripheral leukocytes into the brain parenchyma. These cells have different roles and time course in the progression of ischemic cerebral injury (Figure 3).

Microglia were first described in 1919 by a Spanish physician Pío del Río-Hortega (Sierra *et al.*, 2016) and, depending on the brain region, consist of 5-12% of the total cell population in the mouse brain (Lawson *et al.*, 1990). Microglia originate from the microglial progenitors of the yolk sac which migrate to the brain during early embryonic development and proliferate in the brain to form the adult microglia population (Alliot *et al.*, 1999). It was later confirmed that microglia are, indeed, a distinct population from the hematopoietic stem cells and a unique group of tissue macrophages that are independently maintained in the brain throughout life (Ginhoux *et al.*, 2010;

Schulz *et al.*, 2012; Kierdorf *et al.*, 2013). In contrast, peripheral immune cells are derived from the hematopoietic stem cells of the bone marrow and are constantly renewed. Despite different origins, monocyte-derived macrophages and microglia express the same genes and proteins, therefore making it challenging to reliably distinguish microglia from bone marrow-derived macrophages. Only recently have novel techniques using gene-modified fluorescent reporter mice, chimeras, or fluorescent cell transfer, and the discovery of specific microglia markers (transmembrane protein 119; TMEM119) enabled identification between microglia and monocyte-derived macrophages.

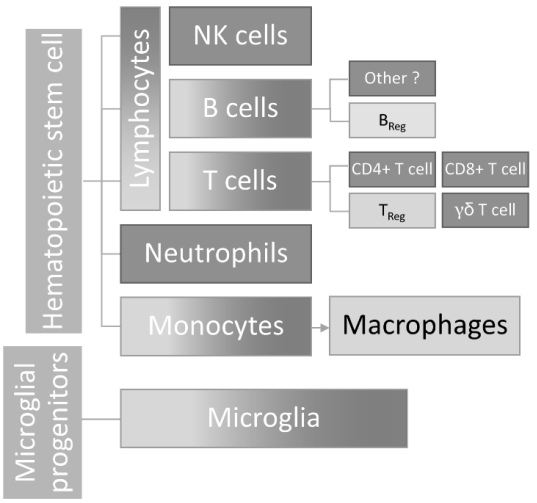

Figure 3. A simplified categorization of the role of major myeloid cell types (microglia, monocytes/macrophages, neutrophils) and lymphocytes in acute ischemic stroke. An overall beneficial role of each cell type in stroke is indicated with green color and a detrimental role with red color. Lymphocytes, neutrophils, and monocytes derive from hematopoietic stem cells of the bone marrow while microglia derive from microglia progenitors of the yolk sac. Monocytes differentiate into macrophages after migration to tissues. Of lymphocytes, regulatory B (B_{Reg}) and T cells (T_{Reg}) have beneficial roles in ischemic stroke, whereas other cell types are considered more harmful. Neutrophils are considered mainly damaging, whereas there is increasing evidence of the beneficial role of monocyte-derived macrophages. Microglia have a complex role with both detrimental and regenerative functions. Macrophages and microglia have an important role in debris removal after ischemia-induced cell death.
NK, natural killer

2.2.1 PERIPHERAL RESPONSES

The peripheral immune system has a prominent role in the post-stroke inflammatory response and stroke pathogenesis. The peripheral inflammatory response to ischemia is induced instantly in the vascular endothelium and platelets by the release of adhesion molecules which attract blood leukocytes

to the endothelial surface at the infarct site and can cause further blockage of blood flow when clustered. Also, thrombin, the activated blood-coagulation factor residing in the ischemia-inducing blood clot, causes chemotaxis, and other proteases of the coagulation cascade can activate the innate immunity complement system. Activation of the complement system has been linked with worse outcomes in ischemic stroke patients (Szeplaki *et al.*, 2009). BBB disruption leads to leukocyte infiltration into the brain parenchyma and conversely, to leakage of cytokines and other inflammatory mediators into the systemic circulation. Also, other blood components enter the brain causing water uptake by osmosis and edema. Monocytes are the major infiltrating cell type (see in chapter 2.2.3) and the relative amount of other leukocytes is significantly smaller.

Inflammatory cytokines increase within a few hours after ischemia-reperfusion injury in mouse blood, spleen, lung, and liver (Offner *et al.*, 2006a; Chapman *et al.*, 2009). In ischemic stroke patients, elevated plasma C-reactive protein (CRP) and cortisol levels and white blood cell count have already been found at admission to the hospital and increased interleukin-6 (IL-6) levels by 24h after symptom onset (Emsley *et al.*, 2003). High plasma levels of IL-6 and CRP in acute ischemic stroke have been associated with larger infarcts and worse neurological outcome (Fassbender *et al.*, 1994a; Smith *et al.*, 2004; Basic Kes *et al.*, 2008), whereas low IL-10 levels were associated with better outcomes (Basic Kes *et al.*, 2008).

After the acute response, ischemic stroke induces depression of the peripheral immune system and infections are a common complication in stroke patients. Immunosuppression may be caused by the release of glucocorticoids and catecholamines after stroke (Fassbender *et al.*, 1994b; Chamorro *et al.*, 2007). Decreased spleen size (Sahota *et al.*, 2013; Chiu *et al.*, 2016), splenic contraction (Vahidy *et al.*, 2016; Zha *et al.*, 2018), and increased apoptosis of lymphocytes (Urra *et al.*, 2009) within the first days after stroke has been observed in patients. In mice, ischemic stroke induces persistent lymphopenia and shrinkage of spleen and thymus caused by increased apoptosis of lymphocytes (Prass *et al.*, 2003; Offner *et al.*, 2006b; Bao *et al.*, 2010). Apoptosis was reversed with a β-adrenergic receptor antagonist and may be caused by overactivation of the sympathetic nervous system (Prass *et al.*, 2003), or alternatively, nuclear protein high motility group box 1 (HMGB1) release from necrotic cells of the infarct to systemic circulation in mice and stroke patients has been suggested to cause immunosuppression (Liesz *et al.*, 2015). In rats, the primary cause for splenic lymphopenia is a catecholamine-induced contraction of the spleen and the release of splenocytes into the systemic circulation (Ajmo *et al.*, 2009; Seifert *et al.*, 2012), highlighting species differences.

2.2.2 INFILTRATION OF LYMPHOCYTES AND NEUTROPHILS

The spleen is the major source of monocytes and lymphocytes and a splenectomy 2 weeks before MCAo decreases infarction size in male (Ajmo *et al.*, 2008; Chauhan *et al.*, 2018), but not in female animals, possibly due to sex differences in regulatory T cells involved in adaptive immunity (Dotson *et al.*, 2015). However, in patients, splenectomy has been associated with an increased risk for stroke (Lin *et al.*, 2015). CD4+ and CD8+ T cells have a deleterious role in acute ischemia-reperfusion injury, and T cell depletion in mice reduces infarct size (Yilmaz *et al.*, 2006; Hurn *et al.*, 2007). Also δγ T cells exacerbate ischemia-reperfusion injury in mice by producing pro-inflammatory IL-17 (Shichita *et al.*, 2009). However, regulatory T cells are neuroprotective in both permanent and transient MCAo in mice by reducing inflammation (Liesz *et al.*, 2009; Li *et al.*, 2013). Also, regulatory B cells have shown benefits in experimental acute ischemic stroke and reduced ischemic injury in mice by releasing IL-10 (Ren *et al.*, 2011; Bodhankar *et al.*, 2013). In patients, low plasma levels of B cells at admission were associated with poor outcome at 3 months post-stroke (Urra *et al.*, 2009). However, B cells have been shown to contribute to the development of long-term cognitive decline after transient MCAo in mice (Doyle *et al.*, 2015). Natural killer cells, also a lymphocyte subtype, exacerbate cerebral ischemic-reperfusion injury in mice within the first 12h (Gan *et al.*, 2014).

Whether neutrophils infiltrate into the brain parenchyma after stroke or are present only in the cerebral vessels has been under debate (Enzmann *et al.*, 2013), but current evidence suggests that neutrophils first accumulate in the leptomeninges and perivascular spaces and thereafter also penetrate into the brain parenchyma in rodent models of permanent and transient cerebral ischemia as well as in ischemic stroke patients (Perez-de-Puig *et al.*, 2015; Otxoa-de-Amezaga *et al.*, 2019a). Neutrophil infiltration into the brain parenchyma takes place within 24-72h after permanent or transient ischemia (Barone *et al.*, 1992; Gelderblom *et al.*, 2009; Otxoa-de-Amezaga *et al.*, 2019a). Significant amounts of granulocytes were also found in the infarcted human brains during the first 1-2 days after stroke (Lindsberg *et al.*, 1996). Neutrophils are considered detrimental in ischemic stroke and increase neuronal damage in animal models (Connolly *et al.*, 1996; Neumann *et al.*, 2015) by secreting proteases toxic to neurons and extracellular matrix components (Stowe *et al.*, 2009; Allen *et al.*, 2012), NO (Garcia-Bonilla *et al.*, 2014), and by physically occluding cerebral capillaries and preventing reperfusion (del Zoppo *et al.*, 1991; Mori *et al.*, 1992). However, there is evidence that neutrophils can be harnessed for neuroprotection, where pharmacologically induced polarization of brain-penetrated neutrophils towards regenerative “N2” type reduced ischemic injury (Cuartero *et al.*, 2013). In ischemic stroke patients, high blood neutrophil and total leukocyte

counts within 24h after symptom onset were associated with larger infarct volumes (Buck *et al.*, 2008).

It was recently reported that in infarcted patient *post mortem* brains only a few lymphocytes and neutrophils were found which may suggest that the contribution of these cell types is minor in patients compared to experimental animals (Zrzavy *et al.*, 2018).

2.2.3 MICROGLIAL ACTIVATION AND INFILTRATION OF MONOCYTE-DERIVED MACROPHAGES

Microglia have an important and constant role in maintaining central nervous system (CNS) homeostasis: microglia are involved in the development, remodeling, function, and plasticity of neuronal networks [see in (Kettenmann *et al.*, 2011)]. Microglia express numerous cell surface receptors that continuously sense molecular cues for changes in physiological conditions. Under pathologic conditions, microglia go through phenotypical changes that are regarded as "microglial activation" even though microglia is never inactive. These changes involve morphological alterations from stationary, highly ramified cells towards motile, amoeboid or round-shaped cells resembling peripheral macrophages, acquiring phagocytic function, and production of secreted molecules that can be pro-inflammatory [e.g. tumor necrosis factor α (TNF-α), IL-1β, IL-6, NO], anti-inflammatory (e.g. IL-10), or pro-regenerative [e.g. VEGF, insulin-like growth factor 1 (IGF-1), BDNF, GDNF]. Reactive microglia and macrophages were suggested to possess two distinct phenotypes, the classic pro-inflammatory/degenerative "M1" phenotype, and the alternative anti-inflammatory/regenerative "M2" phenotype. Recent evidence from single-cell transcriptomics has proven this classification an oversimplification with no relevance to *in vivo* conditions and microglia as well as monocyte-derived macrophages express both "M1" and "M2" markers within the same cell [see in (Ransohoff, 2016)]. The environment has been considered as the primary factor defining the microglia phenotype. However, it was recently suggested that microglia population would consist of different subtypes with intrinsic properties that define microglia phenotype, rather than solely the tissue environment [see in (Stratoulias *et al.*, 2019)].

In the ischemic brain, microglia respond rapidly to danger-associated molecular patterns which are intracellular molecules released from the ischemia-injured cells. Microglial activation has already been observed 30 min after permanent MCAo induction in the mouse brain (Rupalla *et al.*, 1998). However, like neurons, microglia are dependent on ATP and die in the infarct core, and thus, microglia are the defining factor for the peri-infarct region. Microglial degeneration has been observed 4h after transient MCAo (Kato *et*

al., 1996) and significant microglia loss at 12-72h (Lehrmann *et al.*, 1997; Ito *et al.*, 2001; Ritzel *et al.*, 2015).

The role of microglia in ischemic stroke is conflicting. In the hyperacute phase, microglia depletion has been shown to increase lesion size, possibly by many mechanisms, including decreased secretion of growth factors, increased neuronal excitotoxicity and Ca^{2+} dysregulation, and increased neutrophil infiltration (Lalancette-Hebert *et al.*, 2007; Szalay *et al.*, 2016; Otxoa-de-Amezaga *et al.*, 2019b). Considering the role of microglia in maintaining CNS homeostasis, it is not surprising that the depletion of microglia is detrimental. Furthermore, transplantation of exogenous microglia during transient MCAo decreased neuronal damage (Kitamura *et al.*, 2004; Narantuya *et al.*, 2010). Narantyua *et al.* characterized the gene expression profile of transplanted microglia and found that several growth factors and anti-inflammatory cytokines were upregulated after transplantation. However, there are also studies showing that acute inhibition of microglia activation is protective in ischemic stroke (Yrjanheikki *et al.*, 1999; Gelosa *et al.*, 2014) but the interventions studied are rarely specific for microglia and direct effects on other immune cells, glia, and neurons may confound results.

Unlike microglia, macrophages survive in hypoxic conditions by switching to anaerobic metabolism. In experimental stroke models, peripheral monocyte-derived macrophages infiltrate the ischemic core peaking between 3 and 7 days post-stroke (Schroeter *et al.*, 1997; Perego *et al.*, 2011; Gliem *et al.*, 2012; Ritzel *et al.*, 2015; Wattananit *et al.*, 2016). In ischemic stroke patients, macrophage accumulation in the infarct region has been found between days 4.5 and 8.5, with the highest macrophage amount at 17-18 days after stroke (Lindsberg *et al.*, 1996). Using chimeric mice, it was shown that at day 7 after permanent MCAo, most of the myeloid cells in the infarct core are peripheral macrophages and not resident microglia, whereas in the peri-infarct region there was an equal amount of both (Tanaka *et al.*, 2003). However, in transient (30 min) MCAo, more resident microglia than infiltrating monocytes were found in the infarct region (Schilling *et al.*, 2003), possibly reflecting the difference in the severity of stroke.

Infiltration of peripheral monocytes to tissues, including the brain, is C-C chemokine receptor 2 (CCR2)-dependent (Boring *et al.*, 1997; Kuziel *et al.*, 1997; Gliem *et al.*, 2012; Wattananit *et al.*, 2016). Traditionally, infiltrating monocytes have been considered detrimental in ischemic stroke and to enhance inflammation and lesion development. Genetic removal of CCR2 or its ligand monocyte chemoattractant protein 1 [MCP-1; a.k.a C-C motif chemokine ligand 2 (CCL2)] reduced infarct volumes compared to wild type mice (Hughes *et al.*, 2002; Dimitrijevic *et al.*, 2007), whereas a later study with a milder infarct found no difference in infarct development despite decreased leukocyte infiltration (Schilling *et al.*, 2009). MCP-1 and CCR2 also have other functions than direct chemotactic effects and it was postulated that the

protective effect was rather a consequence of an altered cytokine profile than decreased monocyte infiltration (Hughes *et al.*, 2002; Schilling *et al.*, 2009).

Microglia, monocytes, and lymphocytes express CX3C chemokine receptor 1 (CX3CR1) which binds the pro-inflammatory chemokine fractalkine [a.k.a CX3C chemokine ligand 1 (CX3CL1)] (Imai *et al.*, 1997; Harrison *et al.*, 1998). In the periphery, fractalkine is expressed on the endothelium where it functions as an adhesion molecule and chemoattractant for monocytes and lymphocytes (Imai *et al.*, 1997). In the brain, fractalkine is expressed primarily by neurons and the interaction with CX3CR1 seems to be important for the communication between microglia and neurons and the control of microglia activation in homeostatic conditions (Harrison *et al.*, 1998; Cardona *et al.*, 2006). Upon ischemic injury, genetic deletion of CX3CR1 reduced infarct volume in mice and improved short-term behavioral recovery after transient MCAo (Denes *et al.*, 2008; Fumagalli *et al.*, 2013; Tang *et al.*, 2014). CX3CR1 deletion was associated with increased anti-inflammatory markers of microglia/macrophages, reduced levels of pro-inflammatory cytokines, and decreased infiltration of peripheral leukocytes at 72h post-stroke. However, the long-term effects of CX3CR1 deletion after ischemic stroke have not been comprehensively studied and could yield different results. It has been suggested that the CX3CR1-expressing immune cells represent a patrolling, homeostasis-maintaining cell population, whereas the CCR2-expressing cells are pro-inflammatory (Geissmann *et al.*, 2003). Interestingly, in patients, high plasma fractalkine levels were associated with better functional outcome at 6 months after ischemic stroke and with decreased inflammation (Donohue *et al.*, 2012).

To date, there is a lot of evidence that the accumulation of monocyte-derived macrophages to the ischemic area is protective and reduces the ischemic lesion and long-term behavioral impairment *via* production of pro-regenerative and anti-inflammatory molecules (Smirkin *et al.*, 2010; Gliem *et al.*, 2012; Perego *et al.*, 2016; Wattananit *et al.*, 2016). However, there are also contradicting data showing reduced ischemic injury after depletion of peripheral monocytes (Ma *et al.*, 2016). The varying results could be explained by a different method of monocyte depletion, model, and time point of analysis. It was suggested that microglia/macrophages first acquire a neuroprotective phenotype which is then switched to a neurodegenerative phenotype during the first week after ischemic stroke (Perego *et al.*, 2011; Hu *et al.*, 2012). However, in ischemic stroke patients, pro-inflammatory markers were found to dominate in the acute phase, while anti-inflammatory markers were expressed later during infarct resolution (Zrzavy *et al.*, 2018), indicating that clinical findings may not correlate with the animal models. Nevertheless, there is increasing evidence from recent animal studies that pro-inflammatory monocytes change phenotype towards anti-inflammatory/repair-promoting after infiltration into the injured tissue (Chu *et al.*, 2015; Garcia-Bonilla *et al.*,

2016; Miro-Mur *et al.*, 2016) which could imply that the ischemic environment drives a neuroprotective phenotype.

In comparison to microglia, invading monocytes were found to be more efficient in phagocytosis at 72h after transient MCAo, indicating that monocyte-derived macrophages have a prominent role in the early debris clearing (Ritzel *et al.*, 2015). Phagocytic removal of tissue debris from the infarcted brain is vital and a prerequisite for the regenerative processes to take over. However, phagocytosis of viable neurons, phagoptosis, can occur in the ischemic brain when stressed neurons expose cell membrane structures that trigger phagocytosis by macrophages and microglia (Neher *et al.*, 2011; Fricker *et al.*, 2012; Neher *et al.*, 2013). Phagoptosis can enhance neuronal damage and deteriorate behavioral recovery in ischemic stroke (Neher *et al.*, 2013).

Clearly, our knowledge of microglia function is still limited, and thorough investigation into different microglia phenotypes in ischemic stroke may aid in elucidating the role of microglia in stroke pathogenesis. Overall, immunomodulation to support anti-inflammatory and reparative processes and to dampen pro-inflammatory molecules would seem like a potential therapeutic goal instead of focusing on specific immune cell types. Our understanding of stroke-induced inflammation is further complicated by the dual role of cytokines, illustrated by TNF-α which is neuroprotective when in membrane-anchored form and neurodestructive when in a soluble form (Clausen *et al.*, 2014; Madsen *et al.*, 2016).

2.2.4 REMOTE SECONDARY DAMAGE AND LONG-TERM MICROGLIAL ACTIVATION

The amount of activated microglia and macrophages decreases in the ischemic core and peri-infarct area within 1-month post-stroke when the necrotic tissue debris has been removed. However, post-stroke neuroinflammation is long-lasting and activated myeloid cells have been found in the brain for up to six months after transient cerebral ischemia (Justicia *et al.*, 2008; Thored *et al.*, 2009; Anttila *et al.*, 2018). Activated microglia were suggested to promote neurogenesis in the subventricular zone (SVZ) for months after intraluminal MCAo by expressing IGF-1 (Thored *et al.*, 2009).

Due to connecting projection pathways, focal ischemic stroke also induces delayed neuronal damage in regions distal to the infarct (Nagasawa & Kogure, 1990) occurring 1-2 weeks after the initial ischemic insult in rodents, and microglial activation has been shown to precede the neurodegeneration (Korematsu *et al.*, 1995; Rupalla *et al.*, 1998; Dihne & Block, 2001). Microglial activation in the thalamus has been reported already 2-3 days after permanent or transient MCAo (Rupalla *et al.*, 1998; Loos *et al.*, 2003). In a permanent MCAo model, the peak microglial activation in the ipsilateral thalamus was

observed between 2-4 weeks with consistent thalamic neuronal loss at 4 weeks post-stroke (Rupalla *et al.*, 1998). No microglial activation was present anymore at 3 months (Rupalla *et al.*, 1998). Another study using a transient MCAo model reported only few activated microglia in the ipsilateral thalamus at 1 week after the MCAo but large amounts of activated microglia were found at 4 weeks post-stroke (van Groen *et al.*, 2005). This secondary pathology is accompanied by β-amyloid and Ca^{2+} accumulation in the ipsilateral thalamus observable from 1 week up to 9 months after transient MCAo in rat (van Groen *et al.*, 2005; Makinen *et al.*, 2008), but significant β-amyloid accumulation has not been found in the nonhuman primate marmoset nor in ischemic stroke patients (Aho *et al.*, 2006; Lipsanen *et al.*, 2013). However, thalamic atrophy and microglial activation similar to rodents have been observed in patients with middle cerebral artery infarction (Tamura *et al.*, 1991; Pappata *et al.*, 2000). The role of secondary thalamic neurodegeneration in post-stroke functional recovery is unknown but the integrity of thalamic circuitry has been implicated in motor recovery of patients (Binkofski *et al.*, 1996). However, a study by Thiel *et al.* found a positive correlation between distal microglial activation and better functional outcome in patients with subcortical infarcts (Thiel *et al.*, 2010), implying the secondary inflammation may have beneficial effects on recovery.

2.2.5 ASTROCYTES

In addition to microglia, astrocytes are an important regulator of CNS homeostasis and the most abundant cell type in the brain [see in (Sofroniew, 2009; Liu & Chopp, 2016; Pekny *et al.*, 2016; Iadecola, 2017)]. Astrocytes regulate synaptic plasticity and synaptogenesis, ion and neurotransmitter homeostasis, provide metabolic support for neurons, and are an essential part of the neurovascular unit regulating cerebral blood flow to meet the needs of neuronal activity.

Upon injury, the branched astrocytes become rapidly reactive, involving changes in gene expression, cell hypertrophy, and in severe cases proliferation and permanent scar formation around the injury (Sofroniew, 2009). The astrocytic scar is considered an attempt to limit tissue injury and inflammation as it forms a physical barrier between the injured and healthy tissue. In a photothrombotic stroke model, reactive astrogliosis was shown to promote behavioral recovery in wild type mice when compared to genetically modified mice with reduced astrocytic scar formation (Liu *et al.*, 2014). Also in other disease models, attenuated scar formation has led to a worse outcome, including increased inflammation, tissue damage, and lesion size (Sofroniew, 2009). Moreover, reactive astrocytes can limit excitotoxicity by uptake of excess extracellular glutamate, increasing BBB repair, and decreasing

vasogenic edema after cerebral injury (Bush *et al.*, 1999), as well as decrease of oxidative stress (Chen *et al.*, 2001b). However, as for microglia, there is also evidence that decreasing reactive astrogliosis is neuroprotective in experimental ischemic stroke (Adelson *et al.*, 2012). Under some circumstances not well known, reactive astrocytes can exacerbate injury *via* pro-inflammatory cytokine and ROS production, glutamate release, and increased edema formation (Sofroniew, 2009).

Reactive astrocytes also regulate innate immunity by expressing innate immunity receptors, such as Toll-like receptors, and by producing cytokines and chemokines [see in (Farina *et al.*, 2007)]. Reactive astrocytes control the extracellular environment with both degenerative and recovery-promoting effects, and can release pro-inflammatory molecules (e.g. TNF-α, IL-6, IL-1β) especially in the acute phase of stroke but also produce anti-inflammatory [e.g. transforming growth factor β (TGF-β)] and neurotrophic [e.g. BDNF, nerve growth factor (NGF), GDNF, ciliary neurotrophic factor (CNTF), VEGF] molecules (Liu & Chopp, 2016).

Collectively, astrocytes have a complex role in post-stroke neuroinflammation and recovery. In addition to possible detrimental effects, astrocytes are involved in multiple brain repair mechanisms after stroke, including neurogenesis, synaptogenesis, angiogenesis, and axonal remodeling. However, many functions of astrocytes remain still unexplored. Interest in astrocyte-targeted stroke therapies has truly started to emerge only during the last decade and is expected to increase as the knowledge on astrocytes' role in ischemic stroke recovery will be further clarified.

2.3 NALOXONE

(-)-Naloxone is a μ, δ, and κ opioid receptor antagonist and is clinically used for the treatment of opioid overdose (Iijima *et al.*, 1978). The (+) enantiomer of naloxone needs to be synthesized separately and has a very low affinity for opioid receptors — approximately 10,000 times less than the (-) enantiomer (Iijima *et al.*, 1978). Numerous clinical trials in stroke patients have been conducted on the acute effects of (-)-naloxone after the first report in 1981 on the ability of naloxone to transiently reverse neurological deficits in patients with cerebral ischemia (Table 1). Unfortunately, all of these trials were designed based on the assumption of the beneficial effects of opioid receptor antagonism in acute ischemic stroke, and the larger patient studies were finally negative (Federico *et al.*, 1991). However, *in vitro* studies (Table 2) with (-)-naloxone later in the 1990s hinted that it may have anti-inflammatory effects independent from opioid receptors (Das *et al.*, 1995; Kong *et al.*, 1997). Still in the 21st century, the *in vivo* experiments in ischemic stroke models were focused on the acute effects of naloxone (Table 3) and pretreatment with (+)-naloxone was shown to be ineffective in limiting the lesion volume, whereas (-

)-naloxone was effective and the protection was associated with μ opioid receptor antagonism (Liao *et al.*, 2003).

The conclusion of the various clinical studies of acute (-)-naloxone seems to be that (-)-naloxone may be able to transiently reverse neurological deficits when given very early in a mild, transient ischemic attack. The acute treatment does not seem to have an effect on the long-term outcome of ischemic stroke. It has been postulated that acute (-)-naloxone treatment would reverse neurological deficits only in the patients who would eventually recover spontaneously (Hans *et al.*, 1992). Also, care should be taken when interpreting the results of open-label trials as (-)-naloxone showed no significant effects in any of the double-blinded studies.

Table 1. Clinical trials conducted with (-)-naloxone in patients with cerebral ischemia. Time of treatment refers to time passed from symptom onset.

Time of treatment	Dose	Outcome	Reference
Several days	0.4 mg i.v. x 3-8 in 24h-48h	Reversal of neurological deficits in 2/2 patients	(Baskin & Hosobuchi, 1981)
First hours	25.2 mg in 30 min	Reversal of neurological deficits in 2/5 patients	(Perey *et al.*, 1984)
≤3-24h	0.8-1.2 mg i.v. x 2-3 in 10 min intervals	Reversal of neurological deficits 3/13 patients	(Jabaily & Davis, 1984)
≤24h	0.4 mg x 3 i.v. in 5 min intervals	No effect (n=40)	(Perraro *et al.*, 1984)
≤8-60h	0.4-4 mg x 2 i.v. in 1h intervals	No effect (n=15)	(Fallis *et al.*, 1984)
5h-8d	0.4 mg i.v. in 30 min	Reversal of neurological deficits in 4/11 patients	(Bussone *et al.*, 1985)
3 groups: <10 min; <24h; 7-14d	0.8 mg i.v.	Reversal of neurological deficits in 3/3 patients (<10min); 7/20 (<24h); no effect (7-14d)	(Estanol *et al.*, 1985)
<48h	52.3-4 978 mg/24h	Reversal of neurological deficits in 13/27 patients	(Adams *et al.*, 1986)
≤48h	4 mg/kg/15 min i.v. loading dose + 2 mg/kg/h for 24 h	No effect (n=38)	(Olinger *et al.*, 1990)
≤12h	5 mg/kg/10 min i.v. loading dose + 3.5 mg/kg/h for 24h	No effect (n=24)	(Federico *et al.*, 1991)

i.v., intravenous

Table 2. *In vitro* effects of naloxone in neurodegeneration and inflammation.

Enantiomer	Model	Effect	Reference
LPS			
(-)	LPS-treated primary neuron-glia cultures	IL-1β ↓ Microglial activation ↓	(Das *et al.*, 1995)
(-)	LPS-treated primary glial cultures	NO; TNF-α ↓	(Kong *et al.*, 1997)
(+); (-)	LPS-treated primary neuron-glia cultures	Neuronal loss ↓ NO; TNF-α ↓	(Liu *et al.*, 2000b)
(+); (-)	LPS-treated primary neuron-glia cultures	Neuronal loss ↓ Microglial activation ↓ NO; IL-1β ↓ Superoxide production ↓	(Liu *et al.*, 2000a)
(-)	LPS-treated primary neuron-glia cultures	Neuronal loss ↓ ROS; superoxide production ↓	(Qin *et al.*, 2005)
(+)	LPS-treated HEK-TLR4 cells LPS-treated rat microglia	TLR4 signaling ↓ CD11b; IL-1β; IL-6 ↓	(Hutchinson *et al.*, 2008)
(+); (-)	LPS-treated primary neuron-glia cultures; PMA-treated neutrophils	Superoxide production ↓	(Wang *et al.*, 2012)
(+)	LPS-treated microglia/ macrophages	NO, TNF-α ↓ Phagocytosis ↓ NF-κB ↔ IRF3; IFN-β ↓	(Wang *et al.*, 2016a)
Other			
(+); (-)	Aβ-treated primary neuron-glia cultures	Neuronal loss ↓ Superoxide production ↓	(Liu *et al.*, 2002)

Aβ, β-amyloid peptide; CD11b, cluster of differentiation molecule 11b; HEK, human embryonic kidney; IFN-β, interferon β; IL, interleukin; IRF3, interferon regulatory factor 3; LPS, lipopolysaccharide; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; NO, nitric oxide; PMA, phorbol myristate acetate; ROS, reactive oxygen species; TLR4, Toll-like receptor 4; TNF-α, tumor necrosis factor α

Table 3. *In vivo* effects of naloxone in neurodegeneration and inflammation.

Enantiomer	Model	Dose	Effect	Reference
Ischemic stroke				
(-)	90min dMCAo in rat	0.03 mg or 0.3 mg/4h i.c.v infusion 1h before dMCAo	Infarct volume ↓ Antioxidant activity ↓ Pyruvate ↑	(Chen *et al.*, 2000)
		0.3 mg/4h i.c.v. infusion 30min after occlusion	Infarct volume ↓	
(-)	90min dMCAo in rat	0.03 mg or 0.3 mg/4h i.c.v. infusion 1h before dMCAo	Infarct volume ↓ Neuroinflammation ↓	(Chen *et al.*, 2001a)
(-)	90min dMCAo in rat	82.5 nmol/4h i.c.v. infusion 1h before dMCAo	Infarct volume ↓ Neutrophil infiltration ↓	(Liao *et al.*, 2003)
(+)			Infarct volume ↔	
Parkinson's disease				
(+); (-)	Intranigral LPS in rat	1 mg/day s.c. infusion for 6d 24h before LPS	Neuronal loss ↓ Microglial activation ↓	(Liu *et al.*, 2000c)
Neuropathic pain				
(+); (-)	Intrathecal LPS injection in mice	10 ng i.t. 10min before LPS	LPS-induced antianalgesia against morphine ↓	(Wu *et al.*, 2006)
(+); (-)	CCI in rat	20-60 µg/h i.t. infusion 10-14d after CCI	Neuropathic pain ↓ CD11b ↓ GFAP ↔	(Hutchinson *et al.*, 2008)

CCI, sciatic nerve chronic constriction injury; CD11b, cluster of differentiation molecule 11b; dMCAO, distal middle cerebral artery occlusion; GFAP, glial fibrillary acidic protein; i.c.v., intracerebroventricular; i.t., intrathecal; LPS, lipopolysaccharide; s.c., subcutaneous

To date, a lot of interest has emerged towards the non-stereospecific anti-inflammatory effects of naloxone enantiomers. It has been proposed that naloxone inhibits Toll-like receptor 4 (TLR4) signaling (Hutchinson *et al.*, 2008; Wang *et al.*, 2016a) by binding to the TLR4 co-receptor MD2 (myeloid differentiation factor 2) and leading to inactivation of the receptor (Hutchinson *et al.*, 2012; Shah *et al.*, 2016). Activation of TLR4 leads to the production of pro-inflammatory cytokines and interferons *via* signaling cascades inducing nuclear translocation of transcription factors NF-κB and interferon regulatory factor 3 (IRF3) [see in (Wang *et al.*, 2013)]. TLR4 is expressed in innate immune cells, glial cells, and neurons, and genetic removal of TLR4 has been shown to protect from cerebral ischemic injury (Cao *et al.*, 2007; Caso *et al.*, 2007; Tang *et al.*, 2007; Kilic *et al.*, 2008). However, other laboratories have failed to replicate the naloxone-induced TLR4 inhibition and the effects of naloxone on TLR4 remain obscure (Skolnick *et al.*, 2014).

Naloxone enantiomers have also been shown to inhibit superoxide production in several studies (Liu *et al.*, 2000a; Liu *et al.*, 2002; Qin *et al.*,

2005; Wang *et al.*, 2012). The neuroprotective and anti-inflammatory effect of naloxone against lipopolysaccharide (LPS) was lost in the primary cultures of PHOX$^{-/-}$ knockout (ko) mice lacking the catalytic subunit 91 kDa glycoprotein (gp91^{phox}) of NADPH oxidase 2 (NOX2), the ROS-producing enzyme, indicating that functional NOX2 is needed for the neuroprotective effect (Qin *et al.*, 2005). Wang *et al.* showed more specifically that both naloxone enantiomers are able to directly interact with the gp91^{phox} subunit leading to inactivation of NOX2 and reduced superoxide production (Wang *et al.*, 2012). NOX2 is abundantly expressed in microglia and NOX2-mediated ROS production is increased upon microglia activation [see in (Haslund-Vinding *et al.*, 2017)]. Neurons also express NOX2, although to a lesser extent, and may contribute to ROS production in pathophysiological conditions. NOX2 expression is increased after cerebral ischemic injury and shown to exacerbate ischemic damage [see in (Kahles & Brandes, 2013)]. Thus, naloxone-induced NOX2 inhibition could be a potential therapeutic approach in ischemic stroke.

Both naloxone enantiomers decrease microglial activation *in vivo* in the rat CNS (Liu *et al.*, 2000c; Hutchinson *et al.*, 2008). Therefore, the immunomodulatory effects of naloxone could be harnessed for potential stroke therapy but would require treatment during the post-stroke inflammation. Notably, naloxone penetrates the BBB and has favorable pharmacokinetic properties for treating CNS diseases (Suzuki *et al.*, 2010). However, naloxone has a short half-life of 1-2h depending on the route of administration (Krieter *et al.*, 2016), and therefore requires frequent dosing. Now that the non-opioid receptor-dependent effects have been revealed, naloxone's full potential in the treatment of ischemic stroke should be explored.

2.4 CDNF/MANF FAMILY OF PROTEINS: FOCUS ON MANF

Cerebral dopamine neurotrophic factor (CDNF) and MANF form the CDNF/MANF family of proteins. Despite their nomenclature as neurotrophic factors, CDNF and MANF are mostly intracellular proteins located in the ER lumen. The MANF gene was first identified in renal cell carcinoma patients in the 1990s (Shridhar *et al.*, 1996), but the presumed mutations were soon verified as normal polymorphism also found in control cases without cancer (Evron *et al.*, 1997). MANF protein, historically also called arginine-rich protein (ARP), based on the many arginines in the human gene amino (N)-terminus which however, turned out not to be translated, or arginine-rich, mutated in early stage of tumors (ARMET), was first discovered in the early 2000s when it was initially isolated from a rat mesencephalic astrocyte cell line and renamed as MANF (Petrova *et al.*, 2003). MANF is evolutionarily conserved and in addition to vertebrates, MANF homologs have been

identified in invertebrates as well, e.g. in fruit fly *Drosophila melanogaster* (DmMANF), aphid *Acyrthosiphon pisum* (Armet), marine sponge *Suberites domuncula* (SDMANF), and nematode *Caenorhabditis elegans* (Manf-1) (Petrova *et al.*, 2003; Palgi *et al.*, 2009; Wang *et al.*, 2015; Sereno *et al.*, 2017; Bai *et al.*, 2018). Shortly after, CDNF protein was discovered and shares 59% amino acid identity with MANF (Lindholm *et al.*, 2007). CDNF is expressed only in vertebrates and its expression levels are much lower than that of MANF, for example in human sera, CDNF protein levels are 170 times lower than MANF levels (Galli *et al.*, 2019a). To date, MANF is known to be involved in various functions of cellular homeostasis and to have cytoprotective effects in several cell types.

2.4.1 FROM STRUCTURE TO FUNCTION

MANF is an 18 kDa protein (Mizobuchi *et al.*, 2007) with two main structural domains, the N-terminal and carboxy (C)-terminal domain, which are connected with a linker (Hellman *et al.*, 2011). In its structure, MANF has three parts that seem to be particularly important for its function: the N-terminal signal peptide, the cysteine bridge (CXXC motif) in the C-terminal domain, and the ER retention signal RTDL at the end of the C-terminus, making MANF not a classical secretory protein (Figure 4).

The N-terminal domain of MANF is saposin-like and predicted to bind lipids and membranes (Parkash *et al.*, 2009). In the N-terminus, MANF has a 21 amino acid long hydrophobic signal sequence that translocates MANF precursor protein to the ER where this signal peptide is cleaved off to form mature MANF protein (Mizobuchi *et al.*, 2007) (Figure 4). Mature MANF is localized in the ER lumen and not in the ER membrane (Mizobuchi *et al.*, 2007). Proteins are translated in ribosomes in the cytosol or outer membrane of the ER. Secretory proteins, approximately one-third of all translated proteins, are directed into the ER lumen by the signal peptide that allows ER translocation *via* a translocon channel [see in (Braakman & Bulleid, 2011)]. Translocation to the ER lumen enables correct protein folding and modifications needed in the extracellular environment and is followed by transportation of the mature proteins to Golgi for secretion or to membranes.

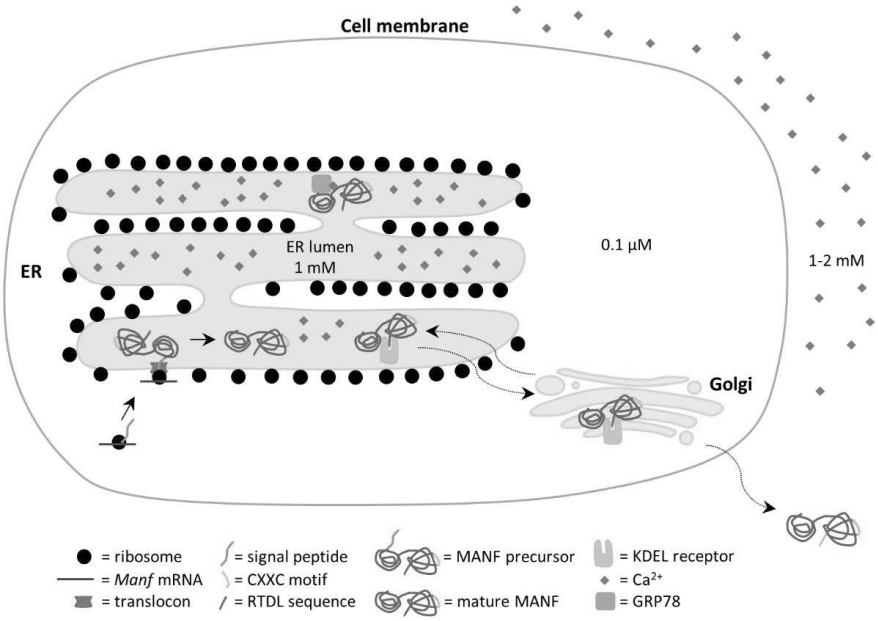

Figure 4. A simplified view of MANF's structure and possible interactions in the ER and Golgi. MANF is directed to the ER by its signal peptide *via* the translocon channel. The signal peptide is cleaved off to form mature MANF protein which resides in the ER luminal space (Mizobuchi *et al.*, 2007). It has been proposed that MANF is held in the ER lumen *via* complex formation with the chaperone GRP78 and Ca^{2+} (Glembotski *et al.*, 2012). MANF is released from the ER upon ER Ca^{2+} depletion. Also KDEL receptors can retain MANF in the ER by binding the RTDL sequence (Henderson *et al.*, 2013). KDEL receptors mediate MANF trafficking between the ER and Golgi (Mizobuchi *et al.*, 2007; Glembotski *et al.*, 2012; Henderson *et al.*, 2013). From Golgi, MANF can be either retrieved back to the ER lumen or secreted into the extracellular space. Approximate Ca^{2+} concentrations in the ER lumen, cytosol, and extracellular space are indicated.

CXXC, two cysteines separated by two other residues; ER, endoplasmic reticulum; GRP78, 78 kDa glucose-regulated protein; KDEL, canonical ER retention signal; RTDL, KDEL-like sequence

The C-terminus of MANF contains an important cysteine bridge that links two helices together (Mizobuchi *et al.*, 2007), and this so-called CXXC motif is needed for both the intracellular and extracellular activity of MANF in mouse primary neuronal cultures as well as in rat *in vivo* model of ischemic stroke (Matlik *et al.*, 2015). Further indicating the significance of the motif, the MANF homolog DmMANF in *D. melanogaster* is inactive without the CXXC motif (Lindstrom *et al.*, 2013). MANF was predicted to have oxidoreductase activity since the CXXC motif is a common structure in oxidoreductases but so far there is no evidence for such function (Mizobuchi *et al.*, 2007; Matlik *et al.*, 2015). Instead, the CXXC motif may be important for achieving the correct conformation of the protein structure (Matlik *et al.*, 2015).

Unlike trophic factors, the C-terminus of MANF contains a 4 amino acid long ER retention signal, RTDL, resembling the canonical ER retention signal KDEL which binds to the KDEL receptors in the ER and Golgi (Mizobuchi *et al.*, 2007; Glembotski *et al.*, 2012; Henderson *et al.*, 2013). This retrieval sequence mediates the relocation of MANF from Golgi to the ER lumen. MANF can also be transported to the Golgi for secretion (Apostolou *et al.*, 2008).

The MANF gene was found to be related to the UPR (Lee *et al.*, 2003) which occurs as a protective mechanism against the accumulation of unfolded proteins within the ER lumen a.k.a. ER stress. MANF mRNA and protein levels are upregulated by ER stress *via* the ER stress response element II (ERSE-II) in the *Manf* promoter region (Mizobuchi *et al.*, 2007; Apostolou *et al.*, 2008; Tadimalla *et al.*, 2008). The ER lumen is responsible for the correct folding and post-translational modifications of newly synthesized secreted and membrane proteins [see in (Walter & Ron, 2011)]. Disturbances in ER function lead to the accumulation of unfolded and misfolded proteins in the ER lumen and trigger the UPR which is regulated by three main pathways: PERK (double-stranded RNA-activated protein kinase-like ER kinase), IRE1 (inositol requiring enzyme 1) and ATF6 (activating transcription factor 6). When activated, these pathways reduce the ER protein load by regulating proteolysis, translation, and mRNA degradation in the ER. Despite UPR being a cytoprotective mechanism, it can lead to apoptosis if ER stress is prolonged, particularly in cells that are secreting substantial amounts of proteins. However, it is not well known whether prolonged ER stress plays a role in neurodegeneration.

Endogenous MANF is secreted from cultured cells as it can be detected in the cell culture medium, and the secretion is highly enhanced by ER Ca^{2+} depletion but not other types of ER stress (Petrova *et al.*, 2003; Apostolou *et al.*, 2008; Tadimalla *et al.*, 2008; Glembotski *et al.*, 2012). It has been shown that removal of the RTDL sequence increases MANF secretion from neuronal and non-neuronal cells in basal conditions (Glembotski *et al.*, 2012; Oh-Hashi *et al.*, 2012; Henderson *et al.*, 2013). However, overexpression of the ER chaperone GRP78 (78 kDa glucose-regulated protein), one of the targets of UPR-regulator ATF6, was able to retain MANF in non-neuronal cells even when the RTDL sequence was deleted, implying an RTDL-independent interaction between MANF and GRP78 (Oh-Hashi *et al.*, 2012). Indeed, GRP78 together with Ca^{2+} was found to form a complex with MANF that retains MANF in the ER lumen, and the ER Ca^{2+} depletion-induced MANF secretion was not dependent on the RTDL sequence in non-neuronal cells (Glembotski *et al.*, 2012). However, Henderson *et al.* showed that the RTDL sequence was, indeed, needed for ER retention and secretion of proteins in response to ER Ca^{2+} depletion in a neuronal cell line (Henderson *et al.*, 2013). These data imply that the regulation of MANF secretion may be cell type-specific, possibly due to differences in ER structure and KDEL receptor

subtypes between cells. Furthermore, it has been implicated that all ER luminal proteins containing the ER retention signal are secreted upon ER Ca^{2+} depletion in neuronal cells (Trychta *et al.*, 2018).

The physiological responses to increased MANF secretion upon ER lumen Ca^{2+} depletion are unknown. Also upon ischemic stroke, ER Ca^{2+} is depleted, releasing MANF to the extracellular space. The released MANF could act as an emergency signal for myeloid cells and modulate the phenotype of these cells and the recruitment of phagocytic cells to the injury area. These views are discussed more in chapter 2.4.4.

2.4.2 EXPRESSION OF MANF

MANF protein is widely expressed in different tissues including the brain (Figure 5), and highly expressed particularly in secretory tissues, such as pancreas, and in the immune system-related tissues, such as bone marrow, lymph node, and spleen in human and mouse (Mizobuchi *et al.*, 2007; Lindholm *et al.*, 2008; Uhlen *et al.*, 2015; Danilova *et al.*, 2019b). This is logical, as MANF has been shown to have a role in protein folding homeostasis (Yan *et al.*, 2019). In the healthy mouse brain, MANF protein is expressed mainly in neurons but *Manf* mRNA levels are also high in astrocytes, microglia, oligodendrocytes, and endothelial cells (Lindholm *et al.*, 2008; Zhang *et al.*, 2014). MANF protein expression is highest during postnatal development on postnatal days 3-6 in mice, decreasing thereafter and is lowest in the adult (Wang *et al.*, 2014). Cerebral MANF expression has been observed already during early embryonic development, mRNA at embryonic day (E) 12.5 and protein at E14, in mice (Lindholm *et al.*, 2008). It was recently shown that MANF protein expression decreases with age in the fruit fly and mouse tissues, and in human serum (Sousa-Victor *et al.*, 2019). Decreased MANF expression could predispose for many age-related diseases, such as ischemic stroke and Parkinson's disease.

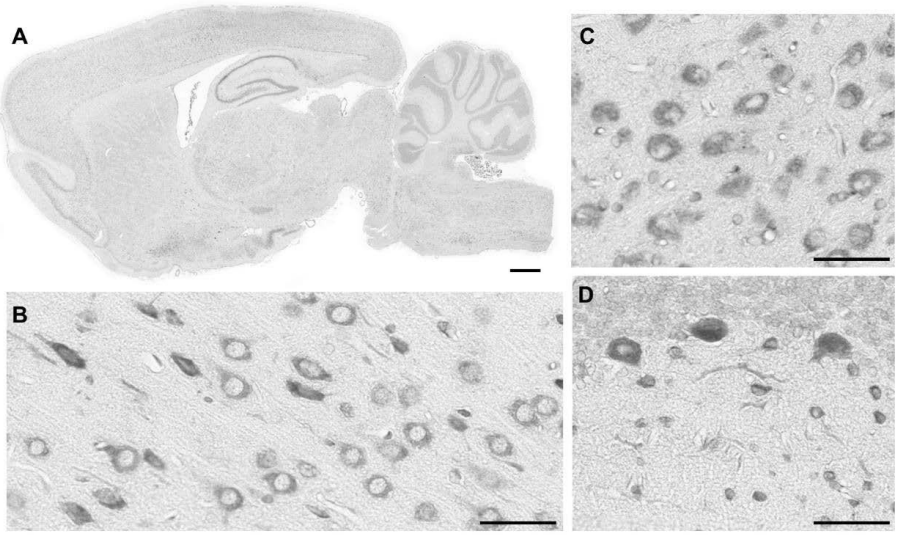

Figure 5. MANF is widely expressed in the brain. **A**: Sagittal view of the anti-MANF immunostained adult rat brain. 40x magnification of the **B**: cortex, **C**: thalamus and **D**: cerebellum. Scale bar is 1000 µm in A and 50 µm in B-D. Unpublished result.

MANF expression is upregulated upon ER stress *in vitro* (Mizobuchi *et al.*, 2007; Apostolou *et al.*, 2008) and *in vivo* after various injuries in different tissue and cell types. In the brain, short (10 min) global ischemia transiently increased neuronal *Manf* mRNA levels at 24h in the rat hippocampus (Lindholm *et al.*, 2008). Transient focal ischemia increased neuronal MANF protein expression at 2-48h post-MCAo in the rat ischemic cortex, especially in the peri-infarct region, while expression in the infarct core was low (Apostolou *et al.*, 2008; Yu *et al.*, 2010). Interestingly, in the same MCAo model, Shen *et al.* showed that MANF protein is upregulated not only in neurons of the ischemic cortex but also in the CD68+ microglia/macrophages and in oligodendrocytes at 24h post-stroke (Shen *et al.*, 2012). Minor MANF expression was also found in astrocytes in the ischemic cortex but not in the non-ischemic brain (Shen *et al.*, 2012). In the CD68+ cells of the ischemic cortex, MANF expression colocalized with GRP78 (Shen *et al.*, 2012). Increase in MANF protein expression, mainly in neurons, has been recently shown to also occur in hemorrhagic stroke models at 3-72h after injury induction (Xu *et al.*, 2018; Li *et al.*, 2019). Also, 24h after traumatic brain injury (TBI) both protein and *Manf* mRNA levels were elevated around the cerebral contusion in rat and human (Li *et al.*, 2018). However, especially in the TBI samples, the quality of the immunostainings could have been improved and, surprisingly, only little basal MANF expression was found in the rat and human brain (Li *et al.*, 2018). In addition, status epilepticus was shown to increase neuronal *Manf* mRNA levels at 2-24h after seizure in mice (Lindholm *et al.*, 2008), and transgenic mice with cerebral β-amyloid deposits had elevated neuronal

MANF expression, together with increased GRP78 and C/EBP homologous protein (CHOP) expression, at 4-6 months of age (Xu *et al.*, 2019).

In the heart, permanent myocardial ischemia increased MANF levels in the area surrounding the infarct at 4-14 days after the injury (Tadimalla *et al.*, 2008). Hypoxia increased *Manf* mRNA and protein levels in rat primary retinal ganglion cells 24-48h after hypoxia induction with cobalt chloride (Gao *et al.*, 2016). In patients with spleen trauma, MANF protein was expressed heavily in the spleen plasma cells that synthesize and secrete large amounts of antibodies and some MANF+ cells also expressed markers of the UPR (Liu *et al.*, 2015). Plasma cells are differentiated B cells and the ER is known to expand upon plasma cell differentiation and is regulated by the UPR pathways (Zhu *et al.*, 2019). MANF protein expression was also found in a few macrophages and T cells but not in undifferentiated B cells (Liu *et al.*, 2015).

Despite the high number of MANF expression studies, often proper controls verifying the specificity of the antibody have been missing. Also, long-term studies of cerebral MANF expression after ischemia and in ischemic stroke patient samples are lacking, thus leaving our knowledge of endogenous MANF expression pattern in ischemia incomplete.

2.4.3 THERAPEUTIC EFFECTS OF MANF

There is a vast amount of evidence that MANF is important for cell homeostasis and able to protect neurons and also other cell types from injuries. At first, MANF was found to specifically promote the survival of dopaminergic neurons (Petrova *et al.*, 2003), but to date it has become clear that MANF also has multiple other effects. MANF has been implicated in the regulation of ER stress, inflammation, apoptosis, neuronal differentiation and migration, as well as energy homeostasis and metabolism (Figure 6). Many of MANF's effects may have not yet been discovered as the putative plasma membrane receptor mediating MANF's effects, if there is such, is still obscure. The studies revealing MANF's role in different disease models are summarized in table 4.

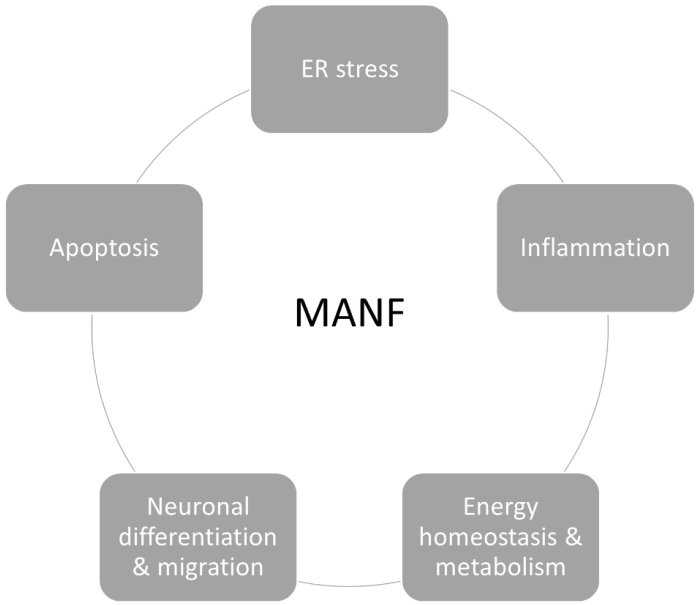

Figure 6. Potential therapeutic targets of MANF.
ER, endoplasmic reticulum

Effects of intracellular MANF

Intracranial delivery of *Manf* cDNA *via* viral vector before ischemic stroke induction protects from the injury (Airavaara *et al.*, 2010) and more importantly, post-stroke delivery of *Manf* cDNA 2 days after ischemic stroke promotes functional recovery (Matlik *et al.*, 2018). Furthermore, endogenous neuronal *Manf* was shown to protect from permanent ischemia using *Nestin*$^{Cre/+}$::*Manf*$^{flox/flox(fl/fl)}$ mice with conditional deletion of *Manf* from neural lineage cells (Matlik *et al.*, 2018). Endogenous *Manf* has also been shown to be important for neuronal differentiation and migration during mouse cortical development (Tseng *et al.*, 2017).

In several organisms, MANF has been shown to be important for either the development or maintenance of dopaminergic neurons. In *D. melanogaster*, the *Manf* homolog *DmManf* is needed for the maintenance of dopaminergic neurites and survival of dopaminergic neurons, and full deletion of *DmManf* is embryonically lethal (Palgi *et al.*, 2009). In the *DmManf* deficient embryos, genes related to ER stress were upregulated and metabolism-related genes downregulated (Palgi *et al.*, 2012). In zebrafish, knockdown of *Manf* during development leads to a reduced number of dopaminergic neurons (Chen *et al.*, 2012). In *C. elegans*, removal of the *Manf* homolog *Manf-1* induces ER stress and degeneration of dopaminergic neurons with aging (Richman *et al.*, 2018), but in young adults, *Manf-1* does not seem to be needed for dopaminergic neurons' survival (Hartman *et al.*, 2019). Intracellular overexpression of *Manf* *via* an adeno-associated viral (AAV) vector protects the dopaminergic cell

bodies of substantia nigra and is able to regenerate the tyrosine hydroxylase-positive fibers in the striatum in the 6-hydroxydopamine (6-OHDA) model of Parkinson's disease even when AAV-MANF is injected into the striatum 10 days after the toxin (Hao *et al.*, 2017). Intracellular overexpression of MANF also protects the SH-SY5Y neuroblastoma cell line from 6-OHDA-induced apoptosis and ER stress *in vitro* (Hao *et al.*, 2017). Interestingly, MANF protein levels were recently found to be increased in Parkinson's disease patients' sera (Galli *et al.*, 2019a).

In the pancreas, MANF is needed for the survival and maintenance of β cells and removal of *Manf* from mouse β cells results in diabetes (Lindahl *et al.*, 2014; Danilova *et al.*, 2019a). Without *Manf* the β cells die due to increased ER stress and apoptosis and decreased proliferation (Lindahl *et al.*, 2014; Danilova *et al.*, 2019a). Viral delivery of *Manf* cDNA protects mouse pancreatic β cells from streptozotocin-induced cell death and enhances β cell proliferation *in vivo* compared to mice injected with *red fluorescent protein* cDNA (Lindahl et al., 2014). In humans, MANF protein levels have been shown to be increased in the serum of newly diagnosed diabetic patients (Galli *et al.*, 2016; Wu *et al.*, 2017).

As an indication of the widespread physiological role of MANF, it was shown that hypothalamic MANF is involved in the regulation of food intake and body weight in mice, and intracellular MANF may have a role in insulin signaling by regulating an ER-localized kinase PIP4k2b (phosphatidylinositol 5-phosphate 4-kinase type-2 beta) (Yang *et al.*, 2017). Overexpression of MANF in the mouse hypothalamus increased food intake and body weight, whereas removal of *Manf* from the hypothalamus had the opposite effect (Yang *et al.*, 2017). Furthermore, fasting increased MANF protein and gene expression in the mouse hypothalamus (Yang *et al.*, 2017). MANF protein levels were also elevated in the liver of obese mice after diet change and weight loss (Galli *et al.*, 2019b). In humans, fasting was recently found to increase MANF protein concentrations in plasma, and the increased circulating MANF may originate at least partly from the liver (Galli *et al.*, 2019b). In both humans and mice, serum levels of metabolism-regulating hormone adiponectin were negatively correlated with MANF levels, indicating a possible role for MANF in metabolic homeostasis (Galli *et al.*, 2019b). Interestingly, a recent study showed that overexpression of MANF increases the lifespan of *D. melanogaster* and that MANF is able to reverse age-related liver damage in mice (Sousa-Victor *et al.*, 2019). MANF deficiency caused changes in lipid metabolism and lipid accumulation in the mouse liver which could be reversed with MANF overexpression. Increasing MANF by overexpression, or a protein injection, also improved glucose and insulin tolerance in old mice.

Effects of extracellularly applied MANF

Overall, recombinant MANF protein is neuroprotective in cerebral ischemia-reperfusion injury when it is injected intracranially before or a few hours after stroke (Airavaara *et al.*, 2009; Yang *et al.*, 2014; Wang *et al.*, 2016b). Also, post-stroke infusion of recombinant MANF protein to the peri-infarct area 3 days after ischemic stroke promotes functional recovery (Matlik *et al.*, 2018) and even a single intracranial MANF injection 7 days post-stroke is able to alleviate neurological deficits (Anttila *et al.*, 2019). *In vitro,* recombinant MANF protein was able to rescue *Manf* ko mouse primary neuronal stem cells from oxygen-glucose deprivation-induced apoptosis (Tseng *et al.*, 2018). Moreover, after cerebral ischemia in rats, exogenous MANF infusion increased the migration of neural progenitor cells into the infarct region (Tseng *et al.*, 2018). Additionally, recombinant MANF protein protected rats from hemorrhagic stroke (Xu *et al.*, 2018; Li *et al.*, 2019) and TBI (Li *et al.*, 2018). In the 6-OHDA model of Parkinson's disease, recombinant MANF protects the dopaminergic cell bodies of the substantia nigra (Voutilainen *et al.*, 2009; Hao *et al.*, 2017). Recombinant MANF also protects the SH-SY5Y neuroblastoma cell line from 6-OHDA-induced apoptosis *in vitro* (Huang *et al.*, 2016; Hao *et al.*, 2017; Sun *et al.*, 2017; Zhang *et al.*, 2017). Notably, in the study by Hao *et al.* extracellularly applied MANF protein had no effect on UPR markers whereas intracellular overexpression of MANF decreased UPR markers in the 6-OHDA-treated SH-SY5Y cells (Hao *et al.*, 2017).

The cytoprotective effects of MANF are not limited to neuronal cells but recombinant MANF protein also protects rat primary myocardial cells from serum starvation-induced apoptosis *in vitro* (Tadimalla *et al.*, 2008) and mouse myocardial cells from ischemia-reperfusion injury *in vivo* (Glembotski et al., 2012). Also in rat retinal ganglion cells recombinant MANF reduced hypoxia-induced apoptosis *in vivo* and *in vitro* (Gao *et al.*, 2017) and decreased photoreceptor loss (Lu *et al.*, 2018), as well as apoptosis in genetic mouse models of retinal degeneration (Neves *et al.*, 2016). In mouse primary podocytes, a kidney cell population, recombinant MANF was found to decrease ER stress-induced cytosolic Ca^{2+} levels by stabilizing the function of the ryanodine receptor channel in the ER and to reduce apoptosis (Park *et al.*, 2019).

As discussed, extracellularly applied MANF has shown cytoprotective effects both *in vivo* and *in vitro*. However, the mechanism of how extracellular MANF exerts its effects in the cell is yet unknown. Based on studies in primary cortical neurons, KDEL receptors were proposed to function as cell surface receptors for MANF by binding the RTDL motif (Henderson *et al.*, 2013). The data was supported by a study on *D. melanogaster* with knockdown of the KDEL receptor homolog in hemocytes, leading to ablation of the cytoprotective effect of extracellular MANF and, thus, suggesting KDEL receptors are needed for mediating MANF's effects in hemocytes (Neves *et al.*,

2016). However, in the rat ischemic stroke model, the RTDL sequence is not needed for the neuroprotective effect which may imply that KDEL receptors are not the key receptors at least in the case of cerebral ischemia (Matlik *et al.*, 2015). More recently, sulphatides were shown to bind extracellular human MANF and to mediate its endocytosis in a non-neuronal cell line and were thus suggested to function as cell surface receptors (Bai *et al.*, 2018). Furthermore, the cytoprotective effect of extracellular MANF was sulphatide-dependent, and the sulphatide-bound MANF was able to reduce ER stress in both *C. elegans* and in mammalian cells (Bai *et al.*, 2018). Also, human CDNF protein – and green fluorescent protein (GFP) – has been shown to enter the cells *via* endocytosis when injected into the rat brain parenchyma (Matlik *et al.*, 2017). However, CDNF does not bind to sulphatides (Bai *et al.*, 2018) and the mechanism of endocytosis is likely different for CDNF, perhaps representing non-specific uptake since GFP was shown to be similarly taken up by the cells (Matlik *et al.*, 2017).

Signaling pathways activated by MANF

Still, little is known about the signaling pathways MANF may mediate its effects with. The Akt (protein kinase B) prosurvival pathway has been implicated in some studies. The protective effect of recombinant human MANF (rhMANF) protein against 6-OHDA-induced apoptosis in SH-SY5Y cells was dependent on activation of the PI3K/Akt/mTOR pathway (Hao *et al.*, 2017) and against hemorrhagic stroke was suggested to be mediated *via* activation of the Akt/MDM2/p53 pathway (Xu *et al.*, 2018; Li *et al.*, 2019). Activation of these pathways leads to enhanced survival and proliferation signaling and has been implicated in various cancers (Mundi *et al.*, 2016). However, no studies have found any pro-tumorigenic effects by MANF, *vice versa* MANF was recently associated with cancer suppression (Liu *et al.*, 2019), hinting that MANF may not be a particularly strong Akt activator. In neuronal stem cell primary cultures, recombinant MANF, and notably, also intracellular overexpression of *Manf* in neural progenitor cell cultures, was shown to activate STAT3 (signal transducer and activator of transcription 3) which could mediate MANF's effects on neuronal differentiation and migration (Tseng *et al.*, 2018). In these cells, MANF had no effect on Akt activation, indicating it does not function as a classical trophic factor.

Table 4. Effects of exogenous MANF therapy (+) and endogenous MANF deletion (-) in different *in vitro* and *in vivo* models.

Model	MANF therapy	Effect	Reference
Apoptosis			
Stroke and TBI			
Ischemic stroke (90min MCAo in rat)	+ (protein)	Infarct volume ↓ Mortality ↓ Neurological function ↑	(Wang *et al.*, 2016b)
Ischemic stroke (60min dMCAo in rat)	+ (protein)	Infarct volume ↓ Apoptosis ↓ Neurological function ↑	(Airavaara *et al.*, 2009)
Ischemic stroke (60min dMCAo in rat)	+ (AAV)	Infarct volume ↓ Neurological function ↑	(Airavaara *et al.*, 2010)
Ischemic stroke (120min MCAo in rat)	+ (protein)	Infarct volume ↓ Apoptosis ↓ Neurological function ↑	(Yang *et al.*, 2014)
OGD in *Manf*$^{-/-}$ ko mouse primary NSCs	+ (protein)	Apoptosis ↓	(Tseng *et al.*, 2018)
Intracerebral hemorrhage in rat	+ (protein)	Apoptosis ↓ Neurological function ↑ Brain edema; BBB leakage ↓ pAkt; pMDM2; Bcl-2 ↑	(Xu *et al.*, 2018)
Subarachnoid hemorrhage in rat	+ (protein)	Apoptosis ↓ Neurological function ↑ Brain edema; BBB leakage ↓ pAkt; pMDM2; Bcl-2 ↑	(Li *et al.*, 2019)
TBI in rat	+ (protein)	Neurological function ↑ Brain edema; BBB leakage ↓ IL-1β; TNF-α; NF-κB ↓	(Li *et al.*, 2018)
Parkinson's disease			
6-OHDA model in rat	+ (protein)	TH+ cell loss in SN ↓	(Voutilainen *et al.*, 2009)
6-OHDA model in rat	+ (AAV)	TH+ cell loss in SN ↓ TH+ fiber loss in STR ↓	(Hao *et al.*, 2017)
Manf knockdown in zebrafish	-	TH+ cell number ↓	(Chen *et al.*, 2012)
6-OHDA-treated SH-SY5Y cells	+ (AAV) + (protein)	Apoptosis ↓ UPR ↓ Apoptosis ↓ UPR ↔ pAkt; pmTOR ↑	(Hao *et al.*, 2017)
6-OHDA or α-synuclein-treated SH-SY5Y cells	+ (protein*)	Apoptosis ↓ GRP78; *HSP70* ↑	(Huang *et al.*, 2016; Sun *et al.*, 2017)
6-OHDA-treated SH-SY5Y cells	+ (protein)	Apoptosis ↓ pmTOR ↑ Mitochondria function ↑ ROS ↓	(Zhang *et al.*, 2017)
Retina			
Light-induced and genetic retinal damage in mouse	+ (protein)	Apoptosis ↓	(Neves *et al.*, 2016)
Hypoxia in rat primary RGCs	+ (protein)	Apoptosis ↓ CHOP ↓	(Gao *et al.*, 2017)
Chronic ocular hypertension in rat	+ (protein)	Apoptosis ↓	(Gao *et al.*, 2017)

Genetic model of photoreceptor degeneration in rat	+ (protein)	Photoreceptor loss ↓	(Lu *et al.*, 2018)
Heart			
Serum-starved rat primary cardiac myocytes	+ (protein)	Apoptosis ↓	(Tadimalla *et al.*, 2008)
Myocardial ischemia-reperfusion in mice	+ (protein)	Infarct size ↓	(Glembotski *et al.*, 2012)
Diabetes			
Manf$^{-/-}$ ko mice	-	β cell death ↑ β cell proliferation ↓	(Lindahl *et al.*, 2014)
Streptozotocin-induced diabetes	+ (AAV)	β cell death ↓ β cell proliferation ↑	(Lindahl *et al.*, 2014)
Other			
NGF-deprived mouse primary SCG neurons	+ (intracellular plasmid or protein injection) + (protein)	Apoptosis ↓ Apoptosis ↔	(Hellman *et al.*, 2011)
ER stress			
Manf$^{-/-}$ ko mouse	-	UPR pathways ↑	(Lindahl *et al.*, 2014)
Manf knockdown in HeLa cells	-	Cell proliferation ↑ Cell size ↓ Morphological changes ↑ ER stress-induced apoptosis ↑ UPR pathways ↑	(Apostolou *et al.*, 2008)
Overexpression of MANF in U2OS cells	+ (LV)	ER stress-induced apoptosis ↓ Cell proliferation ↓ Cell size ↔	(Apostolou *et al.*, 2008)
Manf-deficient *D. melanogaster*	-	UPR pathways ↑ Lethal	(Palgi *et al.*, 2012)
Manf-deficient *C. elegans*	-	UPR ↑	(Richman *et al.*, 2018; Hartman *et al.*, 2019)
Aβ-treated SH-SY5Y cells	+ (protein* or plasmid)	UPR ↓ Apoptosis ↓	(Xu *et al.*, 2019)
ER-stressed mouse primary podocytes	+ (protein*)	Ca^{2+} levels and UPR ↓ Apoptosis ↓	(Park *et al.*, 2019)
Inflammation			
see chapter 2.4.4			
Neuronal differentiation and migration			
Manf$^{-/-}$ ko mouse	-	Neuronal migration ↓ Neurite outgrowth ↓	(Tseng *et al.*, 2017)
Manf$^{-/-}$ ko mouse primary NSCs	-	Apoptosis ↔ Neurite outgrowth ↓ Neuronal differentiation ↓ UPR ↑ Protein synthesis ↓	(Tseng *et al.*, 2017)
Ischemic stroke (90min dMCAo in rat)	+ (protein)	Neuronal migration ↑	(Tseng *et al.*, 2018)
Manf$^{-/-}$ ko mouse primary NSCs;	+ (protein)	Neuronal and glial differentiation ↑	(Tseng *et al.*, 2018)

SVZ explants	(protein / LV)	Cell migration ↑	
Energy homeostasis and metabolism			
Manf-deficient *D. melanogaster*	-	Metabolism-related genes ↓	(Palgi *et al.*, 2012)
Manf knock-in mouse	+ (transgenic)	Food intake ↑ Body weight ↑	(Yang *et al.*, 2017)
MANF overexpression in mouse hypothalamus	+ (AAV)	Food intake ↑ Body weight ↑	
MANF knockdown in mouse hypothalamus	-	Food intake ↓ Body weight ↓	
Manf$^{+/-}$ mouse	-	Lipid metabolism-related genes, lipid accumulation in the liver ↑	(Sousa-Victor *et al.*, 2019)
Old wt mice	+ (protein or plasmid)	Liver damage ↓ Insulin and glucose tolerance ↑	

6-OHDA, 6-hydroxydopamine; AAV, adeno-associated virus; Aβ, β-amyloid peptide; BBB, blood-brain barrier; Bcl-2, B cell lymphoma 2; CHOP, C/EBP homologous protein; dMCAo, distal middle cerebral artery occlusion; ER, endoplasmic reticulum; GRP78, 78 kDa glucose-regulated protein; HSP70, 70 kDa heat shock protein; ICH, intracerebral hemorrhage; IL, interleukin; ko, knockout; LV, lentivirus; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; NGF, nerve growth factor; NSC, neural stem cell; OGD, oxygen-glucose deprivation; pAkt, phosphorylated protein kinase B; pMDM2, phosphorylated murine double minute 2; pmTOR, phosphorylated mechanistic target of rapamycin; RGCs, retinal ganglion cells; ROS, reactive oxygen species; SAH, subarachnoid hemorrhage; SCG, superior cervical ganglion; SN, substantia nigra; STR, striatum; SVZ, subventricular zone; TBI, traumatic brain injury; TH, tyrosine hydroxylase; TNF-α, tumor necrosis factor α; UPR, unfolded protein response; wt, wild type
*NOTE: High concentration of MANF protein used

Different roles of extracellular and intracellular MANF?

Collectively, the removal of *Manf* results in increased ER stress and UPR. MANF has been shown to interact with the chaperone GRP78 in the ER (Glembotski *et al.*, 2012; Yan *et al.*, 2019). Colocalization of MANF and GRP78 was already shown by Tadimalla *et al.* before the direct interaction was known (Tadimalla *et al.*, 2008). It was suggested that MANF stabilizes the complexes of GRP78 and unfolded proteins in the ER by inhibiting nucleotide exchange on GRP78, and thus has a direct role in maintaining ER protein folding homeostasis (Yan *et al.*, 2019). Studies with mutated MANF protein have revealed that localization to the ER lumen is essential for the anti-apoptotic effect of intracellular MANF in mouse primary neuronal cultures (Matlik *et al.*, 2015). However, the RTDL sequence is not needed for the extracellular activity of recombinant MANF protein after parenchymal injection in the rat *in vivo* model of ischemic stroke (Matlik *et al.*, 2015). These data could imply that interaction in the ER is necessary for cytoprotection by intracellular MANF, whereas the cytoprotective mechanism of extracellular MANF is not mediated

by interaction with the ER molecules. Moreover, extracellularly administered MANF protein does not protect primary superior cervical ganglion (SCG) neurons against apoptosis, but MANF protein injection directly to the cytoplasm does (Hellman *et al.*, 2011). Also, MANF does not bind to the surface or enter the primary SCG neurons (Hellman *et al.*, 2011). Therefore, MANF's function may depend on the cell type and the plasma membrane properties as well since extracellular delivery of MANF protein has been shown to protect other cell types in culture (Table 4). There are also some *in vitro* studies showing ER stress-relieving effects with high doses (5 µg/ml – 2 mg/ml) of recombinant MANF (Park *et al.*, 2019; Xu *et al.*, 2019). Also, recombinant MANF at a high dose of 4 µg/ml has shown to upregulate chaperone GRP78 and 70 kDa heat shock protein (HSP70) levels while protecting a 6-OHDA-stressed neuroblastoma cell line from apoptosis (Huang *et al.*, 2016; Sun *et al.*, 2017), pointing out that the UPR also has a protective role. In general, MANF has more robust effects in the *in vivo* settings than in cultured cells which may imply that an intact tissue environment is important for MANF's therapeutic actions or that the *in vivo* therapeutic effect is originating from cell types other than neurons.

In conclusion, the functional role of intracellular and extracellular MANF may be different and may depend on the cell type. Intracellular MANF in the ER lumen may mainly be a regulator of the ER homeostasis whereas extracellular/secreted MANF may have ER-independent functions in various pathophysiological conditions. Despite the increasing amount of MANF-related studies during recent years, the exact mechanism of MANF's function remains unknown.

2.4.4 IMMUNOMODULATORY EFFECTS OF MANF

A rapidly growing amount of evidence from several different model organisms indicates that MANF is able to modulate inflammation (Table 5). MANF has been shown to downregulate the NF-κB pathway and decrease pro-inflammatory cytokine production *in vitro* in several cell types (Zhao *et al.*, 2013; Chen *et al.*, 2015; Zhu *et al.*, 2016; Cunha *et al.*, 2017; Hakonen *et al.*, 2018; Liu *et al.*, 2019). Upon activation, the transcription factor NF-κB complex translocates from the cytosol to the nucleus where it binds to DNA and initiates cytokine transcription. It was suggested that ER stress and inflammation would induce MANF translocation to the nucleus, mediated by the membrane protein SUMO1 (small ubiquitin-related modifier 1), and allow direct interaction with activated NF-κB (Chen *et al.*, 2015; Liu *et al.*, 2019). In a mechanistic study using the human embryonic kidney cell line, MANF inhibited the binding of NF-κB subunit p65 to the DNA via direct interaction of the MANF C-terminal domain and the DNA-binding domain of p65, and

thus decreased the gene expression of pro-inflammatory cytokines (Chen *et al.*, 2015). Also in the patient liver samples, MANF was shown to colocalize with p65 in the nucleus of hepatoma cells and suggested to reduce NF-κB signaling and suppress cancer (Liu *et al.*, 2019). However, MANF is typically reported to localize in the ER and the prevalence of nuclear localization is unknown.

In *D. melanogaster*, full deletion of the *Manf* homolog *DmManf* induced the upregulation of immune and defense response-related genes (Palgi *et al.*, 2012). In glia-specific *DmManf* knockdown, a new DmMANF+ microglia-like cell type was induced which is interesting since microglia-like cells do not normally exist in *D. melanogaster* (Stratoulias & Heino, 2015). Also in *C. elegans*, the *Manf* homolog *Manf-1* knockdown induced changes in innate immunity-related gene expression and reduced growth in the presence of *E. coli* bacteria (Hartman et al., 2019). In marine sponge *S. domuncula*, the MANF homolog SDMANF was shown to colocalize with the Toll-like receptor, a receptor important for innate immunity (Sereno *et al.*, 2017).

In the dMCAo ischemic stroke model, AAV-MANF treatment transiently increased the number of phagocytic CD68+ cells and the mRNA levels of innate immunity-related genes *EGF module-containing mucin-like receptor 1 (Emr1)* and *complement component 3 (C3)* in the peri-infarct region at post-stroke day 4 (Matlik *et al.*, 2018). Using proteomics, AAV-MANF was shown to downregulate innate immunity proteins, calgranulins A and B (S100A8 and S100A9), in the peri-infarct region and the finding was verified with qPCR (Teppo *et al.*, 2020). S100A8 and S100A9 form a complex called calprotectin complex and have a role in phagocyte recruitment and cytokine production. S100A9 was expressed by neutrophils, indicating MANF could regulate neutrophil function (Teppo *et al.*, 2020). In a rat model of TBI, MANF treatment decreased the levels of pro-inflammatory cytokines around the contusion (Li *et al.*, 2018). In light-induced retinal damage in mouse and *D. melanogaster*, MANF increased the number of alternatively activated pro-regenerative/anti-inflammatory innate immune cells, protected from retinal degeneration, and enhanced integration of transplanted photoreceptors (Neves *et al.*, 2016). Interestingly, the protective effect of recombinant MANF in mouse retina was abolished in CX3CR1 ko mice and MANF failed to induce alternative activation in primary macrophage cultures from these mice, indicating that CX3CR1 is needed for the MANF-induced cytoprotection and immunomodulation (Neves *et al.*, 2016). Similarly, in *D. melanogaster*, hemocyte-specific knockdown of the KDEL receptor abolished MANF's protective and immunomodulatory effect. Hence, at least in the retina of mouse and fruit fly, the cytoprotective effect of MANF was suggested to be mediated by immune cells. In heterozygous MANF ko mice, inflammation in the liver was increased compared to wild type, whereas exogenous MANF therapy in old wild type mice decreased liver inflammation (Sousa-Victor *et*

al., 2019). MANF was recently shown to modulate inflammation in the spleen as well. In macrophage-specific MANF ko mice, the number of pro-inflammatory macrophages was increased in the healthy spleen but when hepatic fibrosis was induced, the number of alternatively activated spleen macrophages increased (Hou *et al.*, 2019).

In patients, *Manf* mRNA levels in leukocytes have been shown to be elevated in inflammatory diseases rheumatoid arthritis and systemic lupus erythematosus (Chen *et al.*, 2015). In addition, immune cell activation after ischemic stroke or systemic LPS has been shown to upregulate MANF in microglia (Shen *et al.*, 2012; Sousa *et al.*, 2018).

There is a link between inflammation and ER stress, and activation of the IRE1α UPR pathway, and possibly other UPR pathways as well, can activate NF-κB [see in (Chaudhari *et al.*, 2014)]. It is thus plausible that MANF could downregulate NF-κB and the downstream cytokine production indirectly by downregulating IRE1α and other UPR pathways. However, it has also been proposed that MANF affects the immune cells in an autocrine/paracrine manner thus inducing a phenotypic shift towards reparative functions (Neves *et al.*, 2016). Collectively, MANF has immunomodulatory effects which may be important for the therapeutic function of MANF in different disease conditions. We hypothesize that when MANF is released from injured cells upon ER Ca^{2+} depletion, the released MANF could modulate the immune cell phenotype and recruitment of phagocytic cells to the injury area.

Table 5. Implications for immunomodulatory effects by MANF.

Model	MANF therapy	Effect	Reference
Ischemia			
OGD in primary rat astrocytes	+ (protein)	IL-1β, IL-6, TNF-α ↓ GRP78, NF-κB p65 ↓	(Zhao *et al.*, 2013)
MCAo in rat	-	MANF ↑ in microglia/macrophages	(Shen *et al.*, 2012)
dMCAo in rat	+ (AAV)	Number of phagocytic cells ↑ *Emr1*; *C3* ↑	(Matlik *et al.*, 2018)
dMCAo in rat	+ (AAV)	S100A8; S100A9 ↓	(Teppo *et al.*, 2020)
Other tissue damage			
Light-induced retinal damage in *D. melanogaster* and mouse	+ (protein)	Alternative activation of innate immune cells ↑	(Neves *et al.*, 2016)
TBI in rat	+ (protein)	IL-1β; TNF-α; NF-κB ↓	(Li *et al.*, 2018)
Old wt mouse	+ (protein or plasmid)	Liver inflammation and damage ↓	(Sousa-Victor *et al.*, 2019)
Liver cancer in human		Colocalization with NF-κB subunit p65 Low MANF levels associated with poor survival	(Liu *et al.*, 2019)

Inflammation			
TNF-α-induced 293T cells	+ (plasmid)	NF-κB ↓	(Chen *et al.*, 2015)
LPS-induced rat primary FLS	+ (protein)	IL-1β, TNF-α ↓	
Cytokine-induced damage in human β cells	+ (protein)	Apoptosis ↓ NF-κB pathway ↓ UPR ↓	(Cunha *et al.*, 2017; Hakonen *et al.*, 2018)
LPS-injected mice		*Manf* ↑ in microglia	(Sousa *et al.*, 2018)
Manf removal			
Manf-deficient *D. melanogaster*	-	Immune and defense response-related genes ↑	(Palgi *et al.*, 2012)
Glial *DmManf* knockdown in *D. melanogaster*	-	Appearance of new DmMANF+ microglia-like cell type	(Stratoulias & Heino, 2015)
Manf-1 knockdown in *C. elegans*	-	Changes in innate immunity-related gene expression Reduced growth in the presence of *E. coli*	(Hartman *et al.*, 2019)
Monocyte-macrophage-specific MANF ko	-	Healthy: Number of M1 macrophages in the spleen ↑ Hepatic fibrosis: Number of M2 macrophages in the spleen ↑	(Hou *et al.*, 2019)
$Manf^{+/-}$ mouse	-	Liver inflammation and damage ↑	(Sousa-Victor *et al.*, 2019)
Hepatocyte-specific *Manf* ko mouse	-	Liver IL-1α, TNF-α ↑	(Liu *et al.*, 2019)
Other			
S. domuncula		SDMANF colocalization with Toll-like receptor	(Sereno *et al.*, 2017)

AAV, adeno-associated virus; C3, complement component 3; dMCAo, distal middle cerebral artery occlusion; Emr1, EGF module-containing mucin-like receptor; FLS, fibroblast-like synoviocytes; GRP78, 78 kDa glucose-regulated protein; IL, interleukin; ko, knockout; LPS, lipopolysaccharide; M1, classically activated; M2, alternatively activated; MAPK, mitogen-activated protein kinase; MCAo, middle cerebral artery occlusion; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; NSC, neural stem cell; OGD, oxygen-glucose deprivation; S100A8, calgranulin A; S100A9, calgranulin B; TBI, traumatic brain injury; TNF-α, tumor necrosis factor α; UPR, unfolded protein response; wt, wild type

2.5 CLINICAL TRIALS IN ISCHEMIC STROKE

As in many neurodegenerative diseases, clinical development of novel therapies for ischemic stroke has been extremely challenging and the stroke research field is infamous for failures in clinical trials. Despite vast efforts, only the thrombolytic agent alteplase has reached clinical use globally. Although not a pharmacological approach, mechanical thrombectomy is also widely used in the hyperacute phase of ischemic stroke in addition to thrombolysis. Additionally, the free-radical scavenger edaravone has been approved for clinical use in the treatment of hyperacute ischemic stroke in Japan in 2001

(Edaravone Acute Infarction Study, 2003; Aoki *et al.*, 2017). Most of the experimental therapies have been focused on neuroprotective strategies and targeting the hyperacute mechanisms of ischemic stroke, such as excitotoxicity, oxidative stress, calcium overload, and apoptosis, aiming to salvage the penumbra tissue and prevent the expansion of the infarct core. However, the therapeutic time window is narrow, a few hours at most, and often not achievable in patients. The preclinical evidence is typically from a very acute time point and some of the clinical trials may have failed because of too delayed treatment (Chamorro *et al.*, 2016).

Experimental models of ischemic stroke are able to depict the major pathophysiological hallmarks of human stroke but these models have many shortcomings as well. Most of the preclinical studies are performed in healthy, young, male animals and do not reflect the heterogenous patient population with comorbidities, such as hypertension and diabetes, and comedication. Usually, the ischemic lesion is standardized as in the MCAo model, unlike in patients with large variability in the lesion size and location. There are also differences in the immune system between rodents and humans, and a surgically induced infarct may further trigger immune responses that confound stroke pathophysiology. Brain and vascular anatomy, as well as functional organization of the brain, differ between species. Therefore, it is important to take these differences into account when translating from bench to bedside.

2.5.1 IMMUNOMODULATORY THERAPIES

The therapeutic molecules investigated in this thesis, MANF and (+)-naloxone, have shown immunomodulatory effects and inflammation is indeed one of the major therapeutic targets in ischemic stroke. To evaluate the potential of these interventions, the main clinical studies focused on anti-inflammation are reviewed (Table 6).

The most positive clinical outcomes have been accomplished with the antibiotic minocycline, the IL-1 receptor antagonist (IL-1ra) anakinra, and the leukocyte inhibitors fingolimod and natalizumab. Several open-label studies have been conducted with minocycline showing beneficial effects on neurological functions (Lampl *et al.*, 2007; Padma Srivastava *et al.*, 2012; Amiri-Nikpour *et al.*, 2015) and meta-analyses support the efficacy of minocycline in the treatment of hyperacute ischemic stroke (Malhotra *et al.*, 2018; Sheng *et al.*, 2018). Anakinra was found to decrease neutrophil, total leukocyte, CRP, and IL-6 levels in the blood, and to improve clinical outcomes in patients with cortical infarcts in the first randomized phase II study (Emsley *et al.*, 2005). Unfortunately, a more recent phase II study suggested a possible interaction between anakinra and rtPA leading to a worse outcome (Smith *et*

al., 2018). A phase II trial on natalizumab, an antibody against the adhesion molecule very late antigen-4 (VLA-4) expressed in T cells and monocytes, did not find an effect on the infarct volume, but patients treated with natalizumab had better functional recovery at one and three months post-stroke compared to placebo (Elkins *et al.*, 2017). Several pilot studies on fingolimod, a lymphocyte inhibitor, have found it to decrease infarct growth and to improve functional recovery either with or without alteplase treatment (Fu *et al.*, 2014; Zhu *et al.*, 2015; Tian *et al.*, 2018).

Despite some positive outcomes in the stroke clinical trials with immunomodulatory drugs, none of the treatments have yet reached the market. However, the confirmed safety and potential efficacy of immunomodulation in stroke patients builds hope that it is a feasible treatment strategy also in the clinics. Additionally, cell-based therapies, such as transplantation of mesenchymal or neural stem cells, are increasingly investigated in the treatment of stroke and immunomodulation has been proposed as one of the mechanisms behind their potential recovery-promoting actions (Trounson & McDonald, 2015; Satani & Savitz, 2016; Liska *et al.*, 2017).

Table 6. The outcome of clinical studies using immunomodulatory therapies in ischemic stroke patients. Time indicates hours from symptom onset until initiation of therapy.

Therapy	Time	Outcome	Reference
Immunosuppressant			
Cyclosporine	≤5h	No effect	(Nighoghossian *et al.*, 2015)
Dexamethasone	<24h	No effect	(Ogun & Odusote, 2001)
Dexamethasone + dextran	24-48h	No effect	(Kaste *et al.*, 1976)
Antibiotic			
Minocycline	≤6-24h	Improved neurological function	(Lampl *et al.*, 2007; Padma Srivastava *et al.*, 2012; Amiri-Nikpour *et al.*, 2015)
Dapsone	≤12h	Improved neurological function	(Nader-Kawachi *et al.*, 2007)
Neutrophil adhesion inhibitor			
CD11b/CD18 antagonist (UK-279,276)	≤6h	No effect	(Krams *et al.*, 2003)
Anti-ICAM-1 antibody (enlimomab)	≤6h	Worse outcome	(Enlimomab Acute Stroke Trial, 2001)
IL-1 receptor antagonist (anakinra)	≤6h	Improved neurological function	(Emsley *et al.*, 2005)
Anti-CD18 antibody (Hu23F2G)	≤12h	No effect	(del Zoppo, 2010)
Leukocyte inhibitor			
Anti-VLA-4 antibody (natalizumab)	≤9h	Improved neurological function	(Elkins *et al.*, 2017)
Sphinogosine-1-phosphate receptor modulator (fingolimod)	7-62h; ≤4.5h; ≤6h	Improved neurological function	(Fu *et al.*, 2014; Zhu *et al.*, 2015; Tian *et al.*, 2018)
Other			
Naloxone	see chapter 2.3		
Paracetamol	<12h	No effect	(den Hertog *et al.*, 2009; de Ridder *et al.*, 2017)

CD, cluster of differentiation; ICAM-1, intercellular adhesion molecule 1; IL, interleukin; VLA-4, very late antigen-4

3 AIMS OF THE STUDY

There is a great need for novel therapies for ischemic stroke since despite decades of research there is still no drug that could promote the functional recovery of patients. The aim of this thesis was to study whether post-stroke treatment with MANF or (+)-naloxone, both associated with immunomodulatory effects, could promote recovery. In the clinics, the therapy can rarely be given preventively and it is, therefore, vital to study drug candidates in the context of post-stroke treatment.

The specific aims of the study were:

1) To characterize the time course of neuroinflammation in the rat dMCAo model and MANF protein expression in the dMCAo model and in the infarcted human brain.

2) To determine whether the secondary pathology of the thalamus in the rat dMCAo model is associated with hyperalgesia and can be rescued with MANF or its homolog CDNF.

3) To investigate whether post-stroke intranasal (+)-naloxone delivery promotes recovery in the rat dMCAo model.

4) To study whether intracranial post-stroke delivery of MANF promotes recovery in the dMCAo model and what is the molecular mechanism behind its action.

5) To examine whether the intranasal delivery of MANF is neuroprotective in the rat dMCAo model.

4 MATERIALS AND METHODS

The methods that were used in the studies are listed in Table 7. A detailed description of the methods can be found in the original publications and their supplementary materials. The methods in which the author was involved in are marked with an asterisk.

Table 7. Methods used in the studies.

Method	Original study
Animal experiments	
distal middle cerebral artery occlusion (dMCAo) model in rat*	I, II, III, IV
dMCAo model in mouse	II, III
Stereotaxic injections*	II, IV
Laser Doppler flowmetry*	III
Intranasal administration of drugs*	I, III
Behavioral assays*	
- Neurological tests	I, II, III, IV
- Cylinder test	II
- Open field test	I, II, III
- Hyperalgesia tests	IV
Evans blue albumin extravasation*	III
Mini-osmotic pump implantation	I, II
Immunological detection	
Immunohistochemistry/Immunofluorescence*	I, II, III, IV
Enzyme-linked immunosorbent assay (ELISA)*	I, III
Imaging and morphometric analyses	
Confocal microscopy*	II, III
Magnetic resonance imaging (MRI)	II
Infarct volume/area analysis*	I, II, III, IV
Cell counting*	I, II, IV
Stereology	I
Cell culture experiments	
Magnetic activated cell sorting (MACS)*	I
Production of viral vectors	II
Others	
Real-time quantitative polymerase chain reaction (qPCR)	II
RNA sequencing and transcriptome analysis	II
^{125}I-labeling of MANF	III

4.1 ANIMALS

Male Sprague Dawley rats and male C57BL/6NHsd mice (Harlan/Envigo, Netherlands) were used for the experiments. In addition, gene-modified *Nestin*$^{Cre/+}$::*Manf*$^{fl/fl}$ male mice with *Manf* lacking from neural lineage cells and control *Manf*$^{fl/fl}$ male mice were used.

The animals were housed in groups of 2-5 under a 12h/12h dark-light cycle with *ad libitum* access to food and water. All the animal experiments were approved by the national Animal Experimental Board (protocol approval numbers ESAVI/5459/04.10.03/2011, ESAVI/7812/04.10.07/2015, and ESAVI/13959/2019) and conducted according to the 3R principles of EU directive 2010/63/EU, and Finnish legislation.

4.2 METHODOLOGICAL CONSIDERATIONS

4.2.1 EXPERIMENTAL FOCAL MODELS OF ISCHEMIC STROKE

Experimental focal ischemic stroke is most typically induced in mouse or rat by MCAo [see in (Carmichael, 2005)]. In the most used model, a coated filament suture is inserted into the carotid artery to block the origin of the middle cerebral artery (Belayev *et al.*, 1996) and can be left in place to induce permanent ischemia, or withdrawn usually after 30-120 min to induce ischemia-reperfusion injury. Depending on the duration of the occlusion, the filament MCAo causes a large lesion between 21-45% of the ipsilateral hemisphere that includes regions of the striatum and cortex, and after 60 min also hypothalamus, thalamus, hippocampus, and midbrain. In humans, a similar sized lesion would be fatal in most cases, and moreover, striatal strokes are rare in patients (Delavaran *et al.*, 2013). A variation of the model was developed by Tamura *et al.* where the occlusion is done by directly ligating a proximal stem of the middle cerebral artery through a craniotomy, leading to more restricted ischemic damage to the cortex and also striatum (Tamura *et al.*, 1981). In the dMCAo model, the distal branch of the middle cerebral artery, responsible for the blood flow to the cortex, is ligated directly through a craniotomy (Chen *et al.*, 1986). To increase the consistency of the lesion, either one or both common carotid arteries can be occluded. The dMCAo model produces a smaller lesion than the filament MCAo since blood flow to the striatum is not blocked and the infarction is restricted to the cortex. In all of these models, ischemia can be either permanent or transient with varying occlusion durations. Mimicking more the human pathophysiology, the embolic MCAo can be caused by either injection of microspheres or macrospheres into the blood circulation (Miyake *et al.*, 1993; Gerriets *et al.*, 2003), or by induction of thromboembolic clots, to block blood flow to the

middle cerebral artery (Zhang *et al.*, 1997). The thromboembolic clot model can be used especially to test new thrombolytic treatments. The downside of thromboembolic models is larger variability and the need to use more animals than in the mechanical occlusion models (Ansar *et al.*, 2014).

In addition to models involving the occlusion of the middle cerebral artery, models with high specificity in size and location of the ischemic lesion have been developed. In the photothrombotic model, a photosensitive dye (Rose Bengal) is injected intravenously or intraperitoneally and irradiated through the skull to induce a small cortical lesion (Watson *et al.*, 1985). The photo-activated dye causes damage to endothelial cells that leads to platelet aggregation and thrombi formation causing interruption of local blood flow. Endothelin-1 is a vasoconstrictor peptide that can be injected stereotaxically into the brain parenchyma to create an ischemic lesion in the area of interest (Sharkey *et al.*, 1993).

4.2.2 DISTAL MIDDLE CEREBRAL ARTERY OCCLUSION (dMCAo) MODEL

We chose to use a focal cortical stroke model by occluding the distal middle cerebral artery as the model produces cortical infarcts of clinically relevant size, has low mortality, and the possibility for long-term follow-up. The more frequently used intraluminal MCAo model produces large striatal infarcts and is associated with high mortality and hyperthermia caused by hypothalamic damage, which may confound results and does not depict common features of human infarcts (Carmichael, 2005). As a limitation, the dMCAo model requires craniotomy which may damage the BBB and influence edema-induced pathophysiology that would occur within the closed skull. Surgical procedures and anesthesia may induce e.g. inflammation and peripheral effects, and sham-operated animals are a valuable control group to recognize confounding factors. Anesthesia can generally be criticized as human strokes rarely occur under anesthesia.

We did not routinely monitor cerebral blood flow (CBF) to confirm successful ligation. CBF monitoring is important, especially when studying neuroprotective treatments that are given before the induction of ischemia and could, therefore, influence the CBF giving a false-positive result. CBF was measured with Laser Doppler Flowmetry in study III where only 1/20 animals did not have significant (<65%) reduction in CBF, indicating that unsuccessful ligation is relatively rare. However, the methods used for CBF monitoring give only an arbitrary estimation of the blood flow and not a true measure.

Overall, ischemic stroke models using mechanical occlusion have been criticized for not reproducing the natural cause of infarct (Hossmann, 2012). Also, reperfusion by occlusion removal is immediate and does not depict the

slow spontaneous or thrombolytics-induced reperfusion in humans, although the mechanical occlusion models could be used to mimic mechanical thrombectomy. Ideally, preclinical testing should be done in models representing the clinical situation, e.g. in thromboembolic models or spontaneous hypertension-induced stroke models, but due to high variability, the use of these models would require high animal numbers and could still produce false-negative results. Also, we have used young healthy animals whereas stroke patients are usually elderly and suffer from comorbidity. However, the dMCAo model is technically challenging to perform in aged animals and the mortality is high. Therefore, it can be considered justified to use the highly reproducible mechanical occlusion models and young animals for initial testing of new therapeutics but the efficacy should be verified in other models and in aged animals later on before proceeding to clinical studies.

4.2.3 DRUG DELIVERY

The drugs were administered either intracranially in a stereotaxic surgery or intranasally (Table 8). Both of these methods require anesthesia for which isoflurane was used. Isoflurane has neuroprotective properties (Sakai *et al.*, 2007), opens the BBB (Tetrault *et al.*, 2008), and activates tropomyosin receptor kinase B (TrkB), the receptor for BDNF, and other intracellular signaling routes (Antila *et al.*, 2017). Therefore, it is vital to include a control group receiving the same amount of isoflurane as the therapeutic group. In study I, an additional no treatment group was included to control for the effect of repeated isoflurane administration during chronic intranasal administration.

When performing stereotaxic injections, it is important to consider the mechanical damage the needle may cause in the brain. The damage may be substantial (Penttinen *et al.*, 2016) and we started to use a thin 33G needle to minimize the mechanical injury. Thin glass capillaries could be another option with less mechanical damage. Intracranial delivery is not optimal for clinical use but in initial preclinical studies, it can be considered reasonable in terms of confirming the access of the investigated drug into the therapeutic target area in the brain.

Besides absorbing systemically from the nasal cavity, intranasally delivered molecules can bypass the BBB and access the central nervous system via several pathways, including the olfactory and trigeminal nerves, vascular and cerebrospinal fluid pathways, and lymphatic system (Thorne *et al.*, 2004; Hanson & Frey, 2008; Lochhead & Thorne, 2012). There is evidence that intranasally delivered peptides reach the CNS in humans (Reger *et al.*, 2008) even though the greater distances and different anatomy of nasal pathways

pose problems for bioavailability of intranasal drugs when comparing to rodents. Also, variability in the absorption efficacy between individual subjects is greater after intranasal delivery than with other delivery methods. Intranasal delivery of naloxone was used as an intranasal formulation (Narcan®) is already on the market in the US. Intranasal delivery of rhMANF was used as an alternative non-invasive administration technique.

Table 8. Study design for therapeutic drug delivery.

Study	Design	Dose	Coordinates A/P; L/M; D/V	Treatment groups
I	Intranasal naloxone delivery twice daily for one week starting on post-stroke day 1	14 x 0.0008 - 0.8 mg/kg	-	No treatment Vehicle (H_20) (+)-naloxone: 0.0008 mg/kg 0.008 mg/kg 0.08 mg/kg 0.32 mg/kg 0.8 mg/kg (-)-naloxone: 0.32 mg/kg
II	Intracranial AAV-MANF injection into the peri-infarct region on post-stroke day 2	2 x 2.5 µl	1.6; 2.2; -5.0 and -0.4; 4.0; -5.0	Vehicle (PBS) AAV7-GFP (1.1 x 10^{13} vg/ml) AAV7-MANF (8.1 x 10^{12} vg/ml)
II	Intracranial infusion of rhMANF into the peri-infarct region starting on post-stroke day 3	3 µg/day for 14 days	−0.5; +1.9; −2.5	Vehicle (PBS) rhMANF
III	Intranasal rhMANF delivery 12h before dMCAo, 15 min before dMCAo and immediately after reperfusion	3 x 7 µg or 3 x 20 µg	-	Vehicle (PBS) rhMANF
IV	Intracranial rhCDNF or rhMANF injection into the thalamus on post-stroke day 7	1 x 10 µg in 4 µl	-3.0; -3.0; -6.0	Vehicle (PBS) rhCDNF rhMANF

A/P, anteroposterior; AAV, adeno-associated virus; dMCAo, distal middle cerebral artery occlusion, D/V, dorsoventral; GFP, green fluorescent protein; L/M, lateromedial; PBS, phosphate-buffered saline; rh, recombinant human

4.2.4 BEHAVIORAL ASSAYS

The behavioral tests assessing functional impairment after ischemic stroke should be chosen based on the location of the ischemic lesion. Since the dMCAo model produces an infarct located on the cortex responsible mainly for sensory and motor functions, we focused on sensorimotor tests. Typically, the tests are based on the asymmetry in sensorimotor functions after a unilateral lesion.

An important issue in rodent models is the rapid recovery of the animals, and behavioral deficits usually resolve within 3-4 weeks in rats (Balkaya *et al.*, 2018). Additionally, compensation may be a problem in long-term behavioral testing and can be erroneously considered as behavioral recovery. For long-term behavioral monitoring of stroke recovery, skilled forelimb reaching tasks, e.g. Monotya's staircase test, are considered the most appropriate for detecting chronic sensorimotor impairments but are time-consuming and require training of the animals (Corbett *et al.*, 2017; Balkaya *et al.*, 2018). In this thesis, only relatively short-term behavioral testing was performed for up to 2-3 weeks post-stroke when the deficits are still clearly present.

Body asymmetry test (Borlongan *et al.*, 1995a) and modified Bederson's score (Bederson *et al.*, 1986b) were used to evaluate neurological deficits as these tests are fast to perform and have been shown to reflect the infarct size (Bederson *et al.*, 1986b; Persson *et al.*, 1989; Shen & Wang, 2010). Other tests used were the cylinder test, which measures the deficits in contralateral paw use, and spontaneous locomotor activity. Spontaneous locomotor activity is more a measure of general animal well-being, and only vertical activity has been shown to correlate with the infarct size (Shen & Wang, 2010).

The body asymmetry test was originally developed by Borlongan and Sandberg for a drug-free evaluation of rotational behavior in the unilateral 6-OHDA lesion model of Parkinson's disease (Borlongan & Sanberg, 1995). The use of the test was soon extended to other toxin models and ischemic stroke (Borlongan *et al.*, 1995a; Borlongan *et al.*, 1995b). In the body asymmetry test, the rat is lifted above the testing table by the tail and the number of contralateral turnings of the head and upper body are counted from 20 trials. The maximum impairment is 20 contralateral turns whereas unlesioned animals typically score 10 contralateral turns by turning in each direction with equal frequency. The body asymmetry has been shown to positively correlate with the infarct volume (Shen & Wang, 2010). The test has been criticized for unpredictable changes in the preferred turning direction (Ingberg *et al.*, 2015), but we have never encountered problems when using the dMCAo model.

The modified Bederson's score is based on scoring neurological deficits according to the 3-point scale: 0 = no observable deficit; 1 = rats show decreased resistance to lateral push; 2 = rats keep the contralateral forelimb to the breast and extend the other forelimb straight when lifted by the tail in addition to behavior in score 1; 3 = rats twist the upper half of their body towards the contralateral side when lifted by the tail in addition to behavior in other scores. The neurological score has been shown to positively correlate with the infarct size (Bederson *et al.*, 1986b; Persson *et al.*, 1989; Rogers *et al.*, 1997).

As a limitation, the neurological tests based on scoring are subjective and should therefore always be performed by the same blinded evaluator. The body asymmetry test or Bederson's score does not measure fine motor skills and the

deficits resolve spontaneously over time, making the tests unsuitable for long-term behavioral monitoring (Balkaya *et al.*, 2018).

The cylinder test is based on spontaneous exploratory behavior in a Plexiglas cylinder, where the number of ipsilateral and contralateral forepaw touches to the cylinder walls are counted during the 5-10 minutes observation period. The test is objective, easy, and relatively fast to perform. The cylinder test is also considered to be suited for long-term assessment of motor function (Schallert *et al.*, 2000) and is not influenced by behavioral compensation (Corbett *et al.*, 2017), however, repeated use of the test can cause habituation and a decrease in exploratory behavior (Balkaya *et al.*, 2018).

4.2.5 IMMUNOHISTOCHEMISTRY AND QUANTITATIVE ANALYSES

For the studies, both paraffin and free-floating sections were used. Embedding in paraffin allows the use of thin 5 µm sections with only one cell layer which enables easy acquirement of high-quality images and clear-cut image analysis, but the technique is laborious. Free-floating cryosections are cut in 40 µm thickness with multiple cell layers which makes one-dimensional image analysis challenging, but the technique is fast and allows efficient processing of large sample numbers.

While using methods based on immunological detection, it is of utmost importance to verify that the antibodies used are specific. This can be done by using proper controls, for example, tissue from ko animals that do not express the antigen of interest, or using preadsorption with the antigen before applying the antibody on the tissue analyzed. Also, a control for unspecific binding of immunoglobulins should be included.

Immunostaining offers some advantage over methods such as flow cytometry and western blot by allowing the analysis of the precise location of the antigen of interest within the tissue and within a cell type. However, quantitative analysis from immunostained tissue is always a mere estimation which is strongly influenced by the number of sections quantified and the method of counting. Moreover, immunohistochemical reaction is not a linear process due to saturation and hence, the intensity of the staining does not necessarily correlate with the amount of the antigen (Corbett *et al.*, 2017). Therefore, immunohistochemical methods can be used for counting the number of immunopositive objects but are not recommended for quantifying the intensity of the staining. Stereology, which allows quantification of 3D material from 2D images, is considered an accurate method for cell counting but is very time consuming and subjective. The method involves random sampling of sites from the tissue of interest, followed by manual recognition and counting of cells in these sites, and finally, the cell numbers per volume are obtained based on the thickness, area, and the number of sections

sampled. Recently, several algorithms for automated analysis of digital images have been developed and shown to highly correlate with the cell numbers counted manually using stereology and offer a high-throughput option for stereology (Penttinen *et al.*, 2016; Penttinen *et al.*, 2018). Without developing an algorithm, digital images of stained sections can be analyzed with different image analysis software (e.g. open-source program ImageJ) that automatically count the number of objects that exceed a certain intensity threshold. The advantage of using these software is that it offers a relatively fast analysis of a high number of samples but requires the careful setting of the intensity threshold versus background staining for obtaining reliable object counts and does not take into consideration the 3D structure of the sample.

4.2.6 INFARCT VOLUME/AREA ANALYSIS

2,3,5-triphenyl-2H-tetrazolium chloride (TTC) staining has been shown to measure infarct size as accurately as histological methods 24h after stroke (Bederson *et al.*, 1986a). The TTC staining method is based on a color reaction where the white TTC is reduced to a red compound (1,3,5-triphenyl formazan) by mitochondrial dehydrogenases. After ischemia-induced mitochondrial damage, the infarct area will remain white. At time points earlier than 24h TTC staining is not reliable as permanent mitochondrial damage has not yet occurred (Bederson *et al.*, 1986a). Also, TTC staining is not suitable for infarct volume analysis at later time points where myeloid cells have invaded the infarct area as it is not a specific marker for neuronal cell death but measures only mitochondrial function.

In the neuroprotection studies, the rats were sacrificed at day (d)2 post-stroke, the brains were sliced into seven 2 mm sections, incubated with 2% TTC for 15 min, and the total infarct volume was counted by multiplying infarct area with the thickness of the section. At later time points, the infarct area was quantified using either paraffin or free-floating coronal sections stained with anti-neuronal nuclei (NeuN) antibody or cresyl violet. The infarct area was recognized as being absent from NeuN- or Nissl-positive neurons and expressed as a percentage of the total area of the section to reduce variance caused by tissue processing. When quantifying infarct volumes, it is important to take into consideration whether edema is present at the time of analysis. Brain edema is most pronounced during the first days after ischemic stroke which is why edema correction should be used when quantifying d2 infarct volumes (Lin *et al.*, 1993). The edema correction was done by measuring the area of healthy tissue on the ipsilateral hemisphere and subtracting it from the contralateral hemisphere.

5 RESULTS

5.1 CHARACTERIZATION OF NEUROINFLAMMATION AND SECONDARY PATHOLOGY OF THE THALAMUS IN THE dMCAo MODEL (I, IV)

To study the effects of immunomodulation, it is vital to know the temporal and spatial characteristics of neuroinflammation in the model used. Therefore, we first characterized the dMCAo model we use to induce a focal cortical stroke. The neurological deficits could be observed up to d28 post-stroke, but at that time point there was already spontaneous recovery in Bederson's score test (IV: Fig. 3B). Histological characterization was performed at d2, d7, d14, d28, d56 and d112 post-stroke.

Morphological activation of Iba1+ (ionized calcium-binding adapter molecule 1; a general marker for microglia/macrophages) cells was observed at d2 post-stroke mainly in the peri-infarct region with few cells expressing the phagocytic marker CD68 (Figure 7A; I: Fig. 2C, D). However, at d7 post-stroke, the ischemic cortex was highly covered by activated CD68+ cells (Figure 7A; I: Fig. 2F). At the same time point, morphological activation of Iba1+ cells was also detected in the ipsilateral striatum and thalamus but few cells were CD68+ (Figure 7A; I: Fig. 2E, F). At d14 and d28 post-stroke the number of CD68+ cells was significantly elevated in the ipsilateral thalamus (Figure 7A-B; I: Fig. 2H, J), and phagocytic cells were observed in the thalamus until post-stroke d112 (Figure 7A-B; I: Fig. 2N).

Astrocytes, identified as expressing glial fibrillary acidic protein (GFAP), were activated in the peri-infarct region starting from d2 post-stroke (Figure 7A; I: Fig. 4B), and in the ipsilateral striatum and thalamus starting from d7 post-stroke (Figure 7A; I: Fig. 4C). Intense thalamic astrogliosis was observed for up to d112 post-stroke (Figure 7A; I: Fig. 4G).

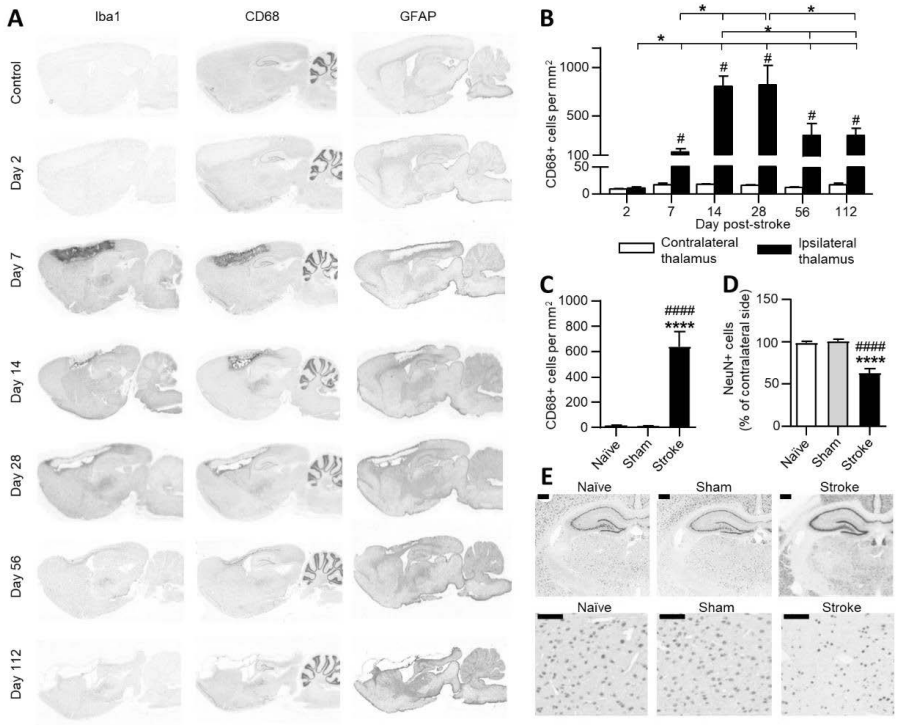

Figure 7. Immunohistochemical characterization of neuroinflammation and secondary neuronal loss in the dMCAo rat model. **A**: Representative images of rat brain sagittal sections at different post-stroke time points immunostained with anti-Iba1, anti-CD68, and anti-GFAP. **B**: Quantitation of CD68+ cells in the thalamus at different time points (n=4). */# p<0.05; * vs. ipsilateral; # vs. contralateral; Kruskal-Wallis test and Mann-Whitney U test. **C**: Quantitation of CD68+ cells and **D**: NeuN+ cells in the ipsilateral thalamus in naïve rats and in rats 28 days after sham or stroke operation; naïve (n=6), sham (n=9), stroke (n=9). ****/#### p<0.0001; **** vs. sham; #### vs. naïve; one-way ANOVA, Bonferroni's post hoc test. **E**: Representative images of anti-NeuN immunostained sections used for NeuN+ cell counting. The data represents the mean ± SEM. Scale bar is 500 µm (E upper row) and 100 µm (E lower row). Figure modified from publications I and IV.

We observed a neuronal loss in the ipsilateral thalamus at d14 (24%; I: Fig. 6B; Figure 13B) and d28 (38%; Figure 7D) post-stroke, simultaneously with the peak in the number of CD68+ cells (Figure 7B-C). However, direct conclusions on the progression of damage cannot be made as the duration of ischemia was different in these d14 and d28 studies, 60 min and 90 min, respectively. We confirmed that sham surgery does not cause any detectable damage in the thalamus (Figure 7C-D). In the striatum, the CD68+ cells were aligned along with axonal bundles and colocalized with myelin basic protein (MBP) (I: Fig. 3), implying phagocytosis of myelin in the degenerating axons connecting the cortex and thalamus.

As the thalamus is an important relay station for pain signals, we also investigated if the secondary thalamic degeneration would induce hyperalgesia. Hyperalgesia has been found after endothelin-1-induced primary ischemic damage to the thalamus (Blasi *et al.*, 2015). However, we found no hypersensitivity toward mechanical or thermal stimuli compared to sham-operated animals at d3, d14 or d28 post-stroke (IV: Fig. 3D-H).

5.2 CHARACTERIZATION OF ENDOGENOUS MANF PROTEIN EXPRESSION AFTER ISCHEMIC STROKE

To study the effects of MANF, it is important to know the temporal and spatial characteristics of endogenous MANF expression in the model used, and in patients for translational relevance. The post-stroke expression of endogenous MANF protein was characterized using immunohistochemistry on tissue samples from the infarcted rat, mouse, and human brains.

5.2.1 ISCHEMIC STROKE INDUCES DELAYED MANF EXPRESSION IN MYELOID CELLS (III)

Similarly to neuroinflammation, we characterized the MANF protein expression pattern in the rat model of dMCAo using immunohistochemistry at d2, d7, d14, d28, d56, and d112 post-stroke. MANF immunoreactivity was increased in the peri-infarct area at all time points (III: Fig. 1b-g). In comparison to the contralateral hemisphere, MANF immunoreactivity was markedly decreased in the infarct core at d2 in rats (Figure 8A, C) but increased in the corpus callosum (III: Fig. 2). However, MANF was strongly upregulated in the infarct core at d7 (Figure 8A) and in the ipsilateral thalamus at d14 (Figure 8A; III: Fig. 1d), coinciding with the peak expression of phagocytic marker CD68 (Figure 7A). The colocalization of MANF and CD68 was verified with double immunofluorescence and confocal microscopy. In the contralateral cortex, MANF colocalized with NeuN (Figure 8B) but at d2 in the ischemic cortex, the NeuN+ cells had lost MANF expression (Figure 8C). However, at d2 and d14, MANF colocalized with CD68 in the ischemic cortex and in the ipsilateral thalamus (Figure 8D, E). Only a few MANF expressing astrocytes (GFAP+ cells) were observed in the ischemic brain.

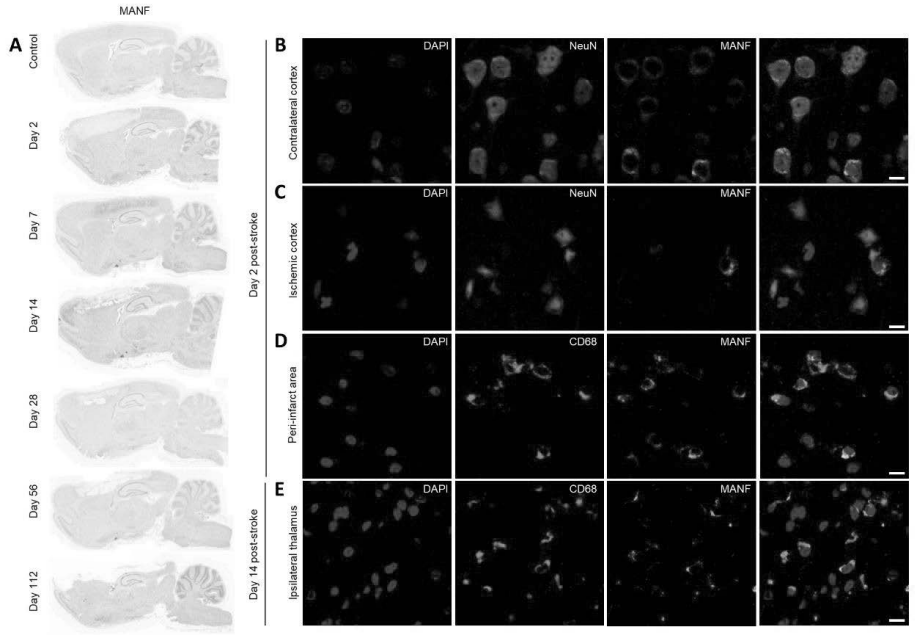

Figure 8. Characterization of endogenous MANF expression pattern in rat brain in the dMCAo model. **A**: Representative images of temporal and spatial MANF expression in sagittal rat brain paraffin sections at different time points after ischemic stroke. **B**: Double immunofluorescence staining of the contralateral cortex, **C**: ischemic cortex, and **D**: peri-infarct area at 2 days post-stroke, and **E**: of ipsilateral thalamus at 14 days post-stroke. NeuN and MANF colocalize in the contralateral (B) but not in the ischemic cortex (C). CD68 and MANF colocalize in the peri-infarct area (D) and in the ipsilateral thalamus (E). Scale bar is 10 µm. Unpublished results.

We investigated whether circulating MANF could be used as a biomarker in ischemic stroke. Endogenous MANF levels were measured from mouse serum at 1h, 6h, 24h, and 48h after permanent dMCAo but we found no significant differences compared to mice without any ischemic damage (Figure 9).

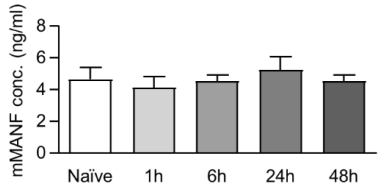

Figure 9. Endogenous MANF serum levels are not altered after permanent cerebral ischemia. Endogenous MANF (mMANF) concentrations were measured from mouse serum at different time points after permanent dMCAo with mouse MANF ELISA (n=3-6). The data represents the mean ± SEM. Unpublished result.

5.2.2 MANF EXPRESSION IS INDUCED IN *Nestin*$^{Cre/+}$::*Manf*$^{fl/fl}$ KNOCKOUT MICE AFTER ISCHEMIC STROKE (III)

Gene-modified *Nestin*$^{Cre/+}$::*Manf*$^{fl/fl}$ mice were used to analyze post-stroke MANF expression in the absence of *Manf* from neural lineage cells. MANF immunoreactivity was lacking in the contralateral cortex of *Nestin*$^{Cre/+}$::*Manf*$^{fl/fl}$ mice (Figure 10B, E) whereas in the *Manf*$^{fl/fl}$ control mice MANF was widely expressed in cortical neurons (Figure 10A). Also, the mRNA levels of *Manf* were very low in the contralateral hemisphere of *Nestin*$^{Cre/+}$::*Manf*$^{fl/fl}$ mice (II: Fig. 4J). *Nestin*$^{Cre/+}$::*Manf*$^{fl/fl}$ mice had larger infarcts on d2 post-stroke compared to control *Manf*$^{fl/fl}$ mice (II: Fig. 4K), indicating a protective role for endogenous neuronal MANF in ischemia.

However, MANF expression was induced in the infarct core, peri-infarct region, and the ipsilateral thalamus of *Nestin*$^{Cre/+}$::*Manf*$^{fl/fl}$ mice at d14 post-stroke (Figure 10B) similarly to control mice (Figure 10A). Double immunofluorescence staining of MANF and Iba1 confirmed the post-ischemic MANF upregulation occurs in microglia/macrophages of the ischemic cortex in the *Nestin*$^{Cre/+}$::*Manf*$^{fl/fl}$ mouse (Figure 10F).

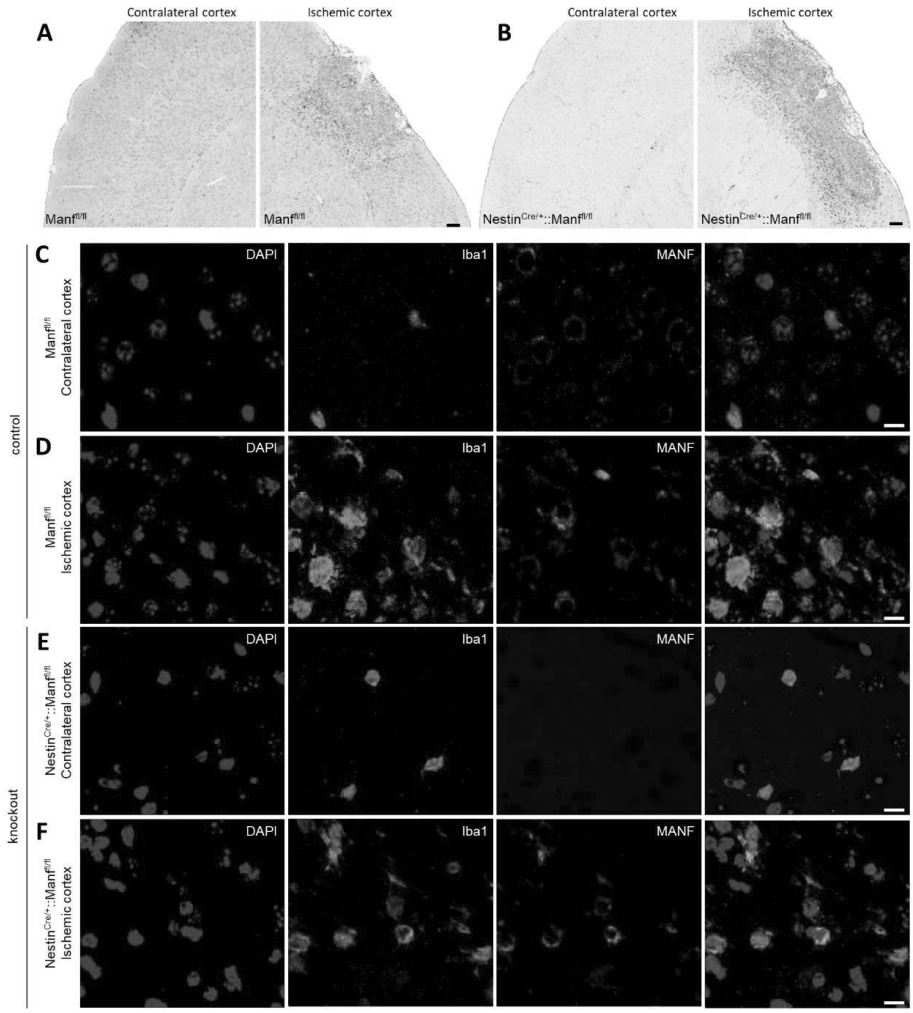

Figure 10. MANF protein expression is induced in microglia/macrophages of the *Nestin*$^{Cre/+}$::*Manf*$^{fl/fl}$ knockout mouse brain after ischemic stroke. Anti-MANF immunostaining of *Manf*$^{fl/fl}$ control (**A**) and *Nestin*$^{Cre/+}$::*Manf*$^{fl/fl}$ knockout (**B**), and anti-MANF and anti-Iba1 double immunofluorescence staining of the contralateral cortex and peri-infarct cortex of *Manf*$^{fl/fl}$ control (**C, D**) and *Nestin*$^{Cre/+}$::*Manf*$^{fl/fl}$ knockout (**E, F**) mouse brain at d14 after permanent dMCAo. A-B: scale bar is 100 µm. C-F: Scale bar is 10 µm. Unpublished results.

5.2.3 ISCHEMIC STROKE INDUCES DELAYED MANF PROTEIN EXPRESSION IN BRAIN IMMUNE CELLS IN HUMANS (III)

We characterized MANF and CD68 expression from consecutive *post mortem* tissue sections of patients deceased approximately 3 days, 1 week, and 2 weeks after ischemic stroke (III: Table 1). In the contralateral side of the infarcted brains, MANF expression was mainly neuronal (Figure 11A, C, E). However, neuronal MANF expression was lost in the infarct core at d3 (Figure 11G), as we also found to occur in the rat dMCAo model. MANF expression remained very low in the ischemic area at 1 week post-stroke (Figure 11I). However, at 2 weeks post-stroke there were high numbers of MANF+ and CD68+ round cells in the infarct region (Figure 11K, L).

Overall, the number and location of MANF+ and CD68+ cells correlated in the infarcted hemisphere. However, we also found CD68+ cells in the contralateral side, especially in the white matter, but the morphology of the cells was more ramified than in the infarcted hemisphere. Some of these white matter cells were also MANF+ but less than in the infarcted hemisphere. Elevated cyclo-oxygenase 2 and TNF-α immunoreactivity, and increased density of intercellular adhesion molecule 1-expressing microvessels have been previously described in these same contralateral hemisphere samples when comparing to non-infarcted control brains (Lindsberg *et al.*, 1996; Sairanen *et al.*, 1998; Sairanen *et al.*, 2001). These data suggest that inflammatory changes also occur in the contralesional human brain after ischemic stroke, and stroke-induced brain herniation and neuroplastic events may influence the contralateral hemisphere. However, CD68+ cells have also been found in the healthy brain, particularly in the white matter, and shown to increase with age (Hendrickx *et al.*, 2017; Hopperton *et al.*, 2018), and the contralateral CD68 expression in the infarcted brains may represent basal levels in the human brain as well.

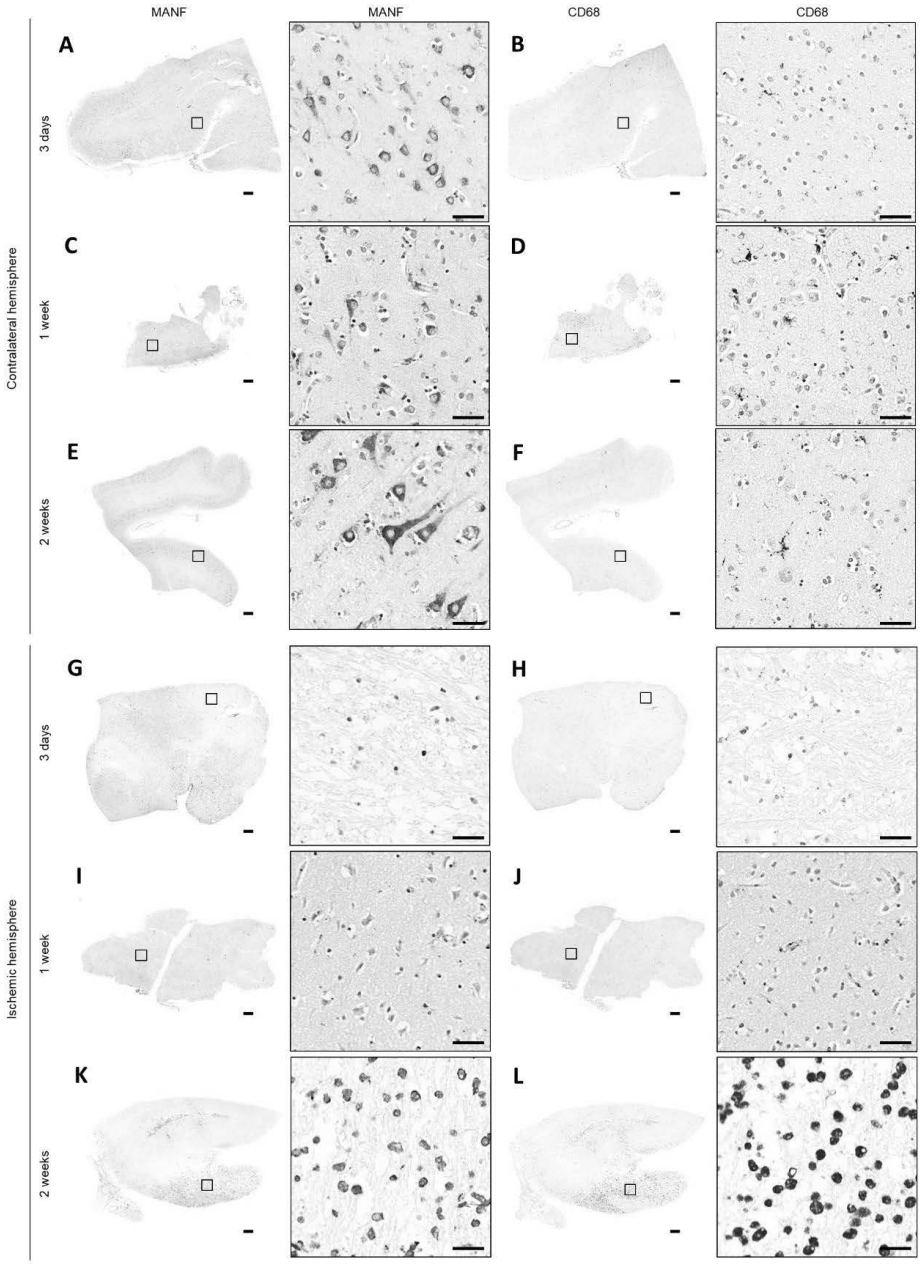

Figure 11. MANF protein expression correlates with CD68 expression at 2 weeks post-stroke in the infarcted human brain. Anti-MANF and anti-CD68 immunostaining of human cerebral tissue from ischemic stroke patients around 3 days, 1 week and 2 weeks post-stroke. **A-F**: Contralateral hemisphere of the ischemic brain. **G-L**: The ischemic region. Scale bar is 1000 µm and 50 µm. Unpublished results.

5.3 EFFECTS OF POST-STROKE INTRANASAL (+)-NALOXONE DELIVERY IN THE dMCAo MODEL

The opioid antagonist naloxone has shown non-stereoselective immunomodulatory effects potentially beneficial in ischemic stroke. Before these effects were known, acute naloxone therapy was in clinical trials and found safe but ineffective. However, naloxone should be further investigated in the context of long-term post-stroke treatment. Furthermore, we concentrated on the (+) enantiomer that has a very low affinity for opioid receptors in order to avoid any potential side effects from opioid receptor antagonism.

5.3.1 POST-STROKE INTRANASAL DELIVERY OF (+)-NALOXONE REDUCES BEHAVIORAL DEFICITS (I)

Intranasal (+)-naloxone was administered twice daily for 7 days starting at d1 post-stroke (Figure 12B). The (+)-naloxone-treated rats had significantly milder neurological deficits at d10 and d14 compared to vehicle and no treatment groups (Figure 12C-D). (-)-Naloxone had a similar effect (Figure 12E-F). The effect of (+)-naloxone was dose-responsive (I: Fig. 5D-E). In addition, spontaneous horizontal activity at d14 was significantly increased in the (+)-naloxone-treated rats compared to no treatment group (I: Fig. 5G).

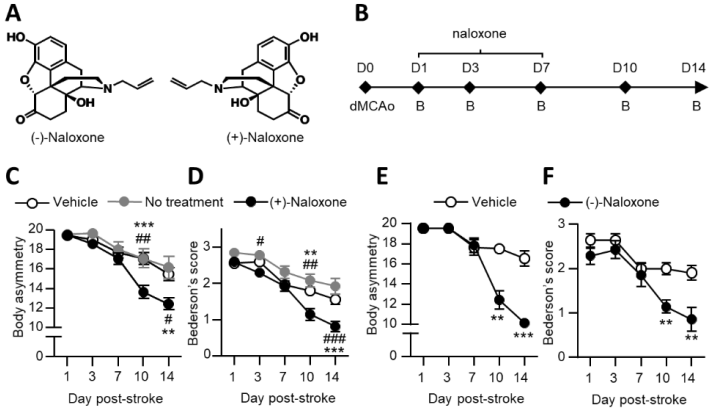

Figure 12. Post-stroke treatment with naloxone reduces behavioral deficits. **A**: (-) and (+) enantiomers of naloxone. **B**: Intranasal naloxone was started on post-stroke day (D) 1 and continued for 7 days twice daily. Behavioral recovery was assessed (B) until day 14. **C**: Body asymmetry test and **D**: Bederson's score after (+)-naloxone treatment. Vehicle (n=25), no treatment (n=13), (+)-naloxone 0.32 mg/kg (n=27), Kruskal-Wallis test and Mann-Whitney U test. **E**: Body asymmetry test and **F**: Bederson's score after (-)-naloxone treatment. Vehicle (n=11), (-)-naloxone 0.32 mg/kg (n=7), Mann-Whitney U test. * vs. vehicle; # vs. no treatment; # ($p<0.05$), **/## ($p<0.01$); ***/### ($p<0.001$). The data represents the mean ± SEM. Figure modified from publication I.

Pretreatment with three doses (0.32 mg/kg) of intranasal (+)-naloxone had no significant effect on the infarct volume (I: Fig. 8A-B). We also investigated if a more delayed intranasal (+)-naloxone therapy, started on d3, would have an effect on behavioral recovery (I: Fig. 8C). There was a trend (p=0.066, Mann-Whitney U test) for milder deficits in the body asymmetry test on d16, but not any statistically significant difference compared to the vehicle group (I: Fig. 8D). In addition, chronic intraventricular delivery of (+)-naloxone starting on d2 post-stroke and continuing for 12 days was investigated (I: Fig. 8E) but there were no differences in the neurological deficits between (+)-naloxone and vehicle groups (I: Fig. 8F). These data imply the therapeutic window is around 24h post-stroke when the infarct is still developing (Li *et al.*, 2000; Liu *et al.*, 2009).

5.3.2 POST-STROKE INTRANASAL DELIVERY OF (+)-NALOXONE REDUCES INFARCT VOLUME AND NEUROINFLAMMATION (I)

The infarct size, secondary neuronal loss of the thalamus, and the amount of Iba1+ cells in the striatum and thalamus were quantified at d14 post-stroke. (+)-Naloxone decreased the infarct size and the neuronal loss in the ipsilateral thalamus (Figure 13A-D) as well as the number of Iba1+ cells in the ipsilateral striatum and thalamus (Figure 13E-G). (+)-Naloxone had no effect on the number of Iba1+ cells in the contralateral striatum (I: Fig. 7B).

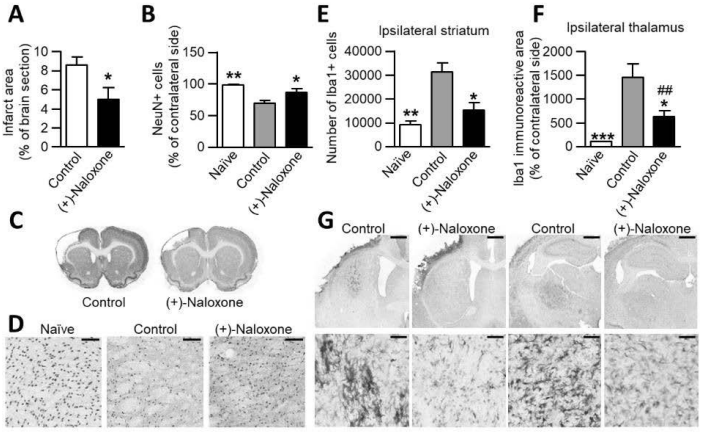

Figure 13. Post-stroke treatment with intranasal (+)-naloxone reduces neuronal loss and neuroinflammation. **A**: Average infarct area (Student's *t*-test) and **B**: number of NeuN+ cells in the ipsilateral thalamus compared to the contralateral side (Kruskal-Wallis test and Mann-Whitney U test) at post-stroke day 14. **C**: Representative images showing the infarct area and **D**: the ipsilateral thalamus in anti-NeuN immunostained sections. **E**: Number of Iba1+ cells in the ipsilateral striatum (one-way ANOVA, Bonferroni's post hoc test) and **F**: Iba1 immunoreactive area in the ipsilateral thalamus compared to the contralateral side (Kruskal-Wallis test and Mann-Whitney U test). **G**: Representative images of ipsilateral striatum and thalamus. Naïve (n=6);

control (n=18); (+)-naloxone (n=10). * vs. control; # vs. naïve; * ($p<0.05$); **/## ($p<0.01$); *** ($p<0.001$). Scale bar is 150 µm (D), 1000 µm (G, upper row) and 50 µm (G, lower row). The data represents the mean ± SEM. Figure modified from publication I.

We conducted an *in vitro* experiment on CD11b+ (marker for microglia and macrophages) cells isolated from the ischemic cortex on d7 post-stroke. After LPS treatment, TNF-α secretion was increased in the isolated CD11b+ cells (I: Fig. 9A), indicating the cells respond to an inflammatory stimulus. The basal TNF-α secretion was measured after overnight treatment with naloxone enantiomers and both enantiomers decreased TNF-α levels in the media by 15% compared to vehicle treatment (I: Fig 9B).

5.4 EFFECTS OF POST-STROKE MANF DELIVERY IN THE dMCAo MODEL

The neuroprotective protein MANF is a promising drug candidate that has shown potential in acute ischemic stroke models. However, in the clinics, the drug treatment cannot be given preventively and it is, therefore, vital to study post-stroke MANF treatment.

5.4.1 POST-STROKE PERI-INFARCT TARGETING OF AAV7-MANF AND rhMANF PROMOTES FUNCTIONAL RECOVERY (II)

AAV7-MANF or AAV7-GFP were intracranially injected using peri-infarct targeting (Matlik *et al.*, 2014) 2 days after the dMCAo. MANF and GFP were robustly expressed in the peri-infarct region already 2 days after the injection i.e. 4 days post-stroke (II: Fig. S7A). The AAV7-MANF-treated rats had milder neurological deficits measured with Bederson's score and body asymmetry test on d4, d7 and d14 post-stroke (II: Figs. 1D-E, S7B-C) and a trend towards better performance in cylinder test (II: Fig. 1F). AAV7-MANF had no effect on the infarct volume evaluated by magnetic resonance imaging (II: Fig. 3D-G) or histology (II: Figs. S3C-D, S8A).

In a separate study, rhMANF was chronically infused to the peri-infarct area for 14 days starting on d3 post-stroke using mini-osmotic pumps. Compared to vehicle, rhMANF-treated rats had milder neurological deficits in body asymmetry test at d24 (II: Fig. 2B) and in cylinder test at d14 post-stroke (II: Fig. 2C).

5.4.2 POST-STROKE PERI-INFARCT TARGETING OF AAV7-MANF INDUCES IMMUNOMODULATORY EFFECTS (II)

AAV7-MANF treatment increased the mRNA levels of innate immunity-related genes *Emr1* and *C3* in the peri-infarct region and the number of CD68 and arginase 1 (Arg1; a marker for alternative activation of myeloid cells) positive cells in the external capsule and dorsal striatum at d4 post-stroke (II: Fig. 4A-I). The effect was transient as the number of CD68+ cells was similar in AAV7-MANF and AAV7-GFP groups at d14 (II: Fig. S9). At d4, there were more MBP and CD68 double-positive cells in the external capsule of AAV7-MANF-treated rats, indicating increased removal of myelin debris (II: Fig. S8C).

AAV7-MANF had no effect on the mRNA levels of TGF-β1 gene *Tgfb1* or *mannose receptor C-type 1 (Mrc1* a.k.a. *CD206)* (II: Fig. S10) which are considered as marker genes for alternative activation of myeloid cells. There were also no differences in the expression of GFAP (II: Fig. S6), MBP (II: Fig. S5), laminin, synaptophysin (II: Fig. S4) or NeuN (II: Fig. S3E) in the peri-infarct area at d14 post-stroke.

5.4.3 POST-STROKE INTRATHALAMIC rhMANF INJECTION ALLEVIATES NEUROLOGICAL DEFICITS (IV)

The delayed secondary damage of the thalamus after cortical stroke offers an intriguing therapeutic target with a wide therapeutic time window, and we aimed to investigate whether the post-stroke recovery can be promoted by modulation of the secondary pathology (Figure 14A). We made a single intrathalamic recombinant MANF protein injection at post-stroke d7 when there is not yet significant neuronal loss in the ipsilateral thalamus (IV; Fig. 4). We included an additional group treated with the MANF homolog CDNF as CDNF has been shown to possess similar neuroprotective effects in acute ischemic stroke as MANF (Zhang *et al.*, 2018) and, also, to modulate inflammation (Cheng *et al.*, 2013; Nadella *et al.*, 2014; Zhao *et al.*, 2014).

Compared to vehicle, the CDNF and MANF-treated rats had milder neurological deficits at d14 (Figure 14B-C). However, there was no difference in the amount of CD68+ cells or neuronal loss in the ipsilateral thalamus at d14 (Figure 14D-E). The infarct volume was confirmed to be similar in all treatment groups (Figure 14F). The recombinant protein distributed widely after the single injection covering the whole ipsilateral thalamus and also parts of the striatum (Figure 14G).

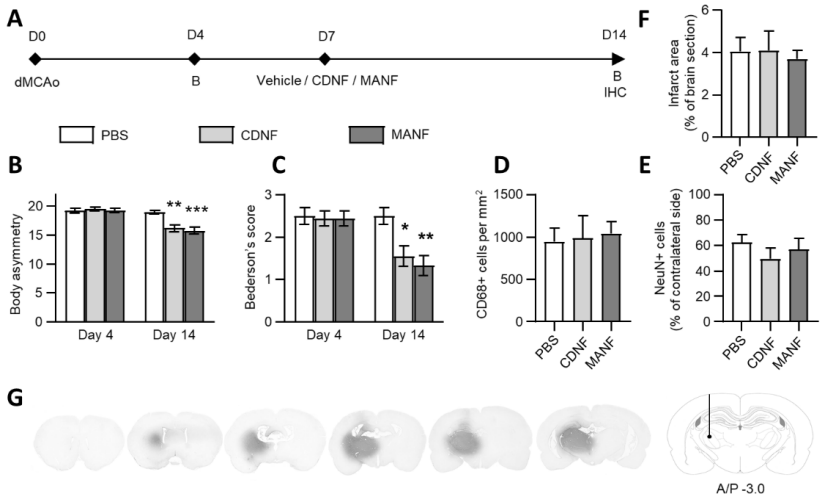

Figure 14. Post-stroke intrathalamic delivery of MANF protein promotes behavioral recovery. **A**: The rats were divided into groups based on behavioral deficits on day 4 post-stroke and recombinant CDNF or MANF protein (10 µg) or vehicle was injected intrathalamically at 7 days post-stroke. The effects on behavioral recovery and secondary pathology were assessed on day 14 post-stroke. **B**: Body asymmetry test and **C**: Bederson's score was performed on days 4 and 14 post-stroke (n=8-9; Kruskal-Wallis test and Mann-Whitney U test). **D**: The number of CD68+ cells and **E**: NeuN+ cells in the ipsilateral thalamus and **F**: infarct volume were quantified by immunohistochemistry at day 14 post-stroke (n=4-6). **G**: The distribution of recombinant human CDNF protein (10 µg) 2h after unilateral intrathalamic injection shown by anti-hCDNF immunostaining, and illustration of the injection site (A/P -3.0; M/L -3.0; D/V -6.0 relative to bregma). * ($p<0.05$); ** ($p<0.01$); *** ($p<0.001$). The data represents the mean ± SEM. B, behavioral assay; D, day post-stroke; dMCAo, distal middle cerebral artery occlusion; IHC, immunohistochemistry. Figure modified from publication IV.

5.5 NEUROPROTECTIVE EFFECTS OF INTRANASAL MANF DELIVERY IN THE dMCAO MODEL

Intracranial delivery is not a plausible administration route in stroke patients when the brain tissue is already compromised and the risk of bleeding is increased due to thrombolysis, and therefore alternative delivery routes for MANF need to be investigated. A proof-of-concept study was performed using the intranasal delivery of recombinant MANF protein for neuroprotection. Theoretically, intranasally delivered molecules can bypass the BBB and access the CNS from the nasal cavity *via* the olfactory and trigeminal nerves, vascular pathways, cerebrospinal fluid pathways, and lymphatic system [see in (Thorne *et al.*, 2004; Hanson & Frey, 2008; Lochhead & Thorne, 2012)]. Using these routes systemic metabolism can also be avoided, offering an advantage over systemic delivery (Meredith *et al.*, 2015).

5.5.1 INTRANASALLY DELIVERED rhMANF REDUCES INFARCT VOLUME AND BEHAVIORAL DEFICITS (III)

Pretreatment with intranasally delivered rhMANF reduced the infarct volume by 30% compared to the vehicle group (Figure 15B), especially in the caudal brain (Figure 15C). The rhMANF-treated rats had significantly milder neurological deficits compared to vehicle-treated rats on d7 and d14 post-stroke measured with body asymmetry test and Bederson's score (Figure 15D-E).

No differences were found between the groups in cerebral blood flow during dMCAo or reperfusion measured with Laser Doppler Flowmetry (Figure 15F). We also tested if intranasal MANF treatment would affect the BBB integrity by analyzing Evans blue albumin extravasation at 48h after dMCAo. There was no difference in Evans blue albumin extravasation into the infarct area between the groups (Figure 15G).

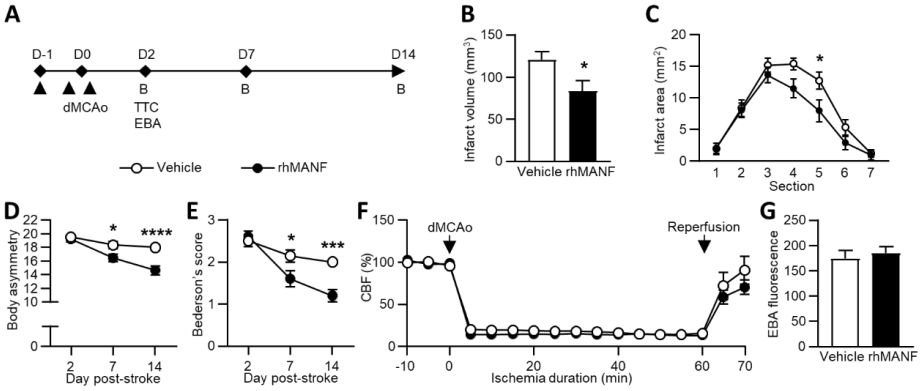

Figure 15. Intranasally delivered MANF protein is neuroprotective. **A**: Recombinant MANF protein (rhMANF; 20 or 60 µg in total) or vehicle was administered intranasally (indicated by black arrows) to rats 12h before dMCAo, immediately before dMCAo, and immediately after reperfusion. Infarct volume and EBA extravasation were measured 2 days post-stroke. Behavioral recovery was monitored for 14 days in a separate experiment. **B**: Infarct volume measured with TTC staining (Student's t-test). **C**: Distribution of infarct area along the rostrocaudal axis (one-way ANOVA, Bonferroni's post hoc test). Vehicle (n=19), rhMANF (n=25). **D**: Body asymmetry test and **E**: Bederson's score (Mann-Whitney U test). **F**: Cortical cerebral blood flow (CBF) was measured with laser Doppler Flowmetry (LDF) before and during ischemia (dMCAo) and after reperfusion. Rats received intranasal rhMANF (n=10) or vehicle (n=9) immediately before LDF measurement. **G**: Average Evans blue fluorescence intensity on the infarct area 2 days post-dMCAo. * ($p<0.05$); *** ($p<0.001$). The data represents the mean ± SEM. B, behavioral assay; D, day post-stroke; dMCAo, distal middle cerebral artery occlusion; EBA, Evans blue albumin; TTC, 2,3,5-triphenyl-2H-tetrazolium chloride staining. Unpublished results.

5.5.2 DISTRIBUTION OF rhMANF AFTER INTRANASAL DELIVERY (III)

The levels of rhMANF in the blood, brain, and liver were analyzed with ^{125}I-labeled MANF 60 min after the intranasal administration (Figure 16A). Approximately 0.4% of the radioactivity of ^{125}I-labeled rhMANF was found in the blood, 0.003% in the brain, and 0.08% in the liver (Figure 16B). The levels of rhMANF in serum and brain were also analyzed with human MANF ELISA, and approximately 0.05% of the total rhMANF dose given was found in the serum (Figure 16C). The levels of rhMANF in the brain were under the detection limit of ELISA, i.e. below 45 pg/ml.

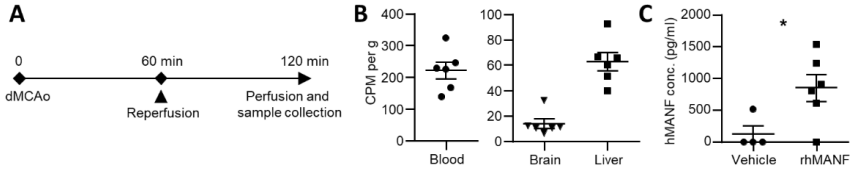

Figure 16. Distribution of rhMANF 1h after intranasal delivery. **A**: ^{125}I-labeled recombinant MANF protein (20 ng; 1081152 CPM) together with unlabeled protein (20 µg), or vehicle was administered to rats intranasally (indicated by black arrow) immediately after dMCAo reperfusion and blood and tissues were collected 60 min later. **B**: Radioactivity counts per minute (CPM) in the rat blood, brain, and liver. **C**: Human MANF concentration in rat serum measured by ELISA (Student's *t*-test). n=4-6; * ($p<0.05$). The data represents the mean ± SEM. dMCAo, distal middle cerebral artery occlusion. Unpublished results.

6 DISCUSSION

6.1 NEUROINFLAMMATION AND ENDOGENOUS MANF EXPRESSION AFTER ISCHEMIC STROKE

We show that MANF expression is induced in activated myeloid cells in the infarcted brains of both rodents and patients. In the infarcted patient brains, the peak expression of phagocytic marker CD68 was observed at two weeks after stroke together with the peak MANF expression in the infarct region. In rats, the peak CD68 and MANF expression in the infarct core occurred earlier, at d7 after stroke, and indeed, it is known that inflammation is more delayed in ischemic stroke patients compared to experimental animals (Lindsberg *et al.*, 1996). The similar post-stroke expression pattern of MANF in patients and experimental animals makes dMCAo a relevant model for studying the role of MANF in inflammation and ischemia.

MANF mRNA is highly expressed in mouse microglia in the healthy brain (Zhang *et al.*, 2014) whereas microglial MANF protein expression has been found to be very low (Shen *et al.*, 2012). We observed some MANF+ microglial cells in the contralateral hemisphere of infarcted rat brains but MANF protein translation appears to be strongly induced after ischemia in the activated myeloid cells that are mainly microglia/macrophages. The role of MANF protein expression in activated myeloid cells and whether it is secreted requires further investigation. However, it is intriguing that a cytoprotective protein is strongly induced in the immune cells upon injury. If MANF is secreted, it could support the injured tissue and provide a pro-reparative environment. Additionally, MANF could be needed in the ER for increased protein production or for ER remodeling during the activation of myeloid cells. The increased protein production in the activated myeloid cells may induce UPR and subsequent MANF translation. This is supported by a study by Shen *et al.* where the ER chaperone GRP78 and MANF were both induced in activated microglia after ischemia (Shen *et al.*, 2012). Also, a general ER stress inducer tunicamycin triggered MANF expression and microglial activation in primary microglia cultures (Shen *et al.*, 2012). Moreover, endogenous and exogenous MANF are important for neuronal migration (Tseng *et al.*, 2017; Tseng *et al.*, 2018), and MANF may be needed in the ER for enabling migration of the activated myeloid cells. MANF may also have a role in phagocytosis as it is expressed particularly in the round, most reactive state of myeloid cells expressing the phagocytic marker CD68, or MANF may be needed for immune cell recruitment. We found that overexpression of MANF in the peri-infarct area increases the number of CD68+ cells in the same region d4 after dMCAo and another study found increased numbers of CD11b+ cells in the damaged

retina after intravitreal rhMANF injection (Neves *et al.*, 2016), supporting MANF's role in immune cell recruitment.

As cerebral MANF protein expression is altered after ischemia in the brain and also likely in infiltrating myeloid cells, we explored whether free circulating MANF could have potential as a biomarker in ischemic stroke diagnostics. However, we found no changes in mouse serum MANF levels during the first two days after permanent ischemic stroke and the data do not thus support the use of circulating free MANF as a potential biomarker in ischemic stroke. The origin of circulating MANF is unknown but at least in the case of ischemic stroke, it does not seem to be released from the brain or from peripheral myeloid cells to the systemic circulation.

We observed long-lasting neuroinflammation especially in the ipsilateral thalamus lasting up to 4 months after dMCAo. We also investigated the secondary neurodegeneration in the thalamus induced by cortical injury and found delayed thalamic neuronal loss occurring between d7 and d14 after dMCAo. As thalamus has an important role in pain perception and direct ischemic injury of the thalamus has been shown to induce thermal hypersensitivity (Blasi *et al.*, 2015), we examined if the secondary thalamic damage is associated with hyperalgesia but found no difference between the stroke and sham animals, implying the damage is not severe enough or is not occurring in the neurons participating in the nociceptive signaling. It is not known whether the secondary thalamic neuronal loss affects post-stroke recovery or whether the persistent presence of activated myeloid cells in the infarcted brain is detrimental or beneficial for the recovery. However, these CD68+ cells express MANF which could implicate they may have recovery-promoting effects.

6.2 POST-STROKE EFFECTS OF NALOXONE

We show intranasal (+)-naloxone treatment started d1 after dMCAo and continued for one week reduced behavioral deficits and decreased neuroinflammation and infarct size. Interestingly, the behavioral effects of (+)-naloxone were not seen until after the treatment was stopped, at d10 and d14 post-stroke, even though we observed a reduction in the infarct volume. The infarct volume was quantified at d14 but it would seem likely that (+)-naloxone had neuroprotective effects leading to reduced lesion size already within the first couple of days after the dMCAo when the infarct is still developing. Therefore, it is surprising that the neurological deficits were similar between the groups until d10 post-stroke. The repeated isoflurane anesthesia required for the intranasal dosing may have masked the effects of (+)-naloxone until the treatment was stopped and the amelioration of neurological deficits could be observed. Another explanation for the delayed behavioral effect could be the drastic decrease in secondary

neuroinflammation and neuronal damage of the thalamus in the (+)-naloxone-treated animals since these secondary pathology events are initiated after d7 post-stroke in the dMCAo model and could hinder the recovery of the control animals.

Naloxone enantiomers inhibit the ROS-producing enzyme NOX2 (Qin *et al.*, 2005; Wang *et al.*, 2012), and NOX2 expression has been shown to be increased in microglia/macrophages in the infarct core and penumbra 16-24h after ischemic stroke (Green *et al.*, 2001; Cooney *et al.*, 2013), around the same time point when we started the intranasal naloxone treatment. Interestingly, NOX2 expression was shown to decrease by d7 in the ischemic brain (Cooney *et al.*, 2013), indicating that we targeted the naloxone treatment for the peak expression period. Although the effects of naloxone on TLR4 are unclear, TLR4 expression has also been shown to increase between 1-22h after ischemic stroke (Hyakkoku *et al.*, 2010). As both NOX2 and TLR4 activation have been shown to increase ischemic injury (Cao *et al.*, 2007; Caso *et al.*, 2007; Tang *et al.*, 2007; Kilic *et al.*, 2008; Kahles & Brandes, 2013), it is likely that the intranasal naloxone treatment started at 16-36h after dMCAo was neuroprotective through inhibition of either one or both of these target proteins. Given the short half-life of naloxone, therapy lasting over the peak microglial activation period may be necessary for the neuroprotective effect.

6.3 POST-STROKE EFFECTS OF MANF

Even though improvement in the neurological deficit scores have been traditionally correlated with infarct volume, post-stroke AAV and recombinant MANF as well as recombinant CDNF treatment were shown to improve the deficits without affecting lesion volume. Currently, the mechanism behind this phenomenon cannot be explained and requires further investigation. In terms of post-stroke AAV7-MANF therapy, thorough histological characterization was performed at d14 but compared to the AAV7-GFP group, no apparent changes were found in astrogliosis, angiogenesis, myelination, or synaptogenesis, the processes associated with post-stroke brain repair mechanisms. However, a transient increase in the number of phagocytic cells and transcript levels of *Emr1* and *C3* was found at d4 in the peri-lesional areas and we hypothesized that enhanced clearance of debris could lead to improved recovery. Better debris clearance could allow improved neuroplasticity and functional changes in the peri-infarct area neurons that cannot be detected with histological methods. C3 has been shown to regulate synaptic plasticity and is involved in immune cell recruitment and phagocytosis (Stephan *et al.*, 2012). During development, removal of C3 results in impaired synaptic connectivity and remodeling (Schafer *et al.*, 2012). Moreover, the cleavage product of C3, C3a, has been shown to increase synaptic plasticity and functional recovery after photothrombotic stroke

(Stokowska *et al.*, 2017). Therefore, the upregulation of *C3* may contribute to the observed functional improvement after AAV-MANF treatment.

Recombinant MANF protein infusion to the peri-infarct area has been shown to increase the migration of neural progenitor cells into the infarct area (Tseng *et al.*, 2018). However, we did not find any differences between the AAV7-MANF and AAV7-GFP groups in the number of mature neurons (NeuN+ cells) in the peri-infarct areas at d14, suggesting that the mechanism of improved recovery after ischemic stroke is not related to neurogenesis in the affected cortex. Though, d14 is a very early time point for neurogenesis and does not rule out the possibility that MANF could affect neurogenesis later on.

As an attempt to modulate the secondary pathology of the thalamus, we gave a single intrathalamic injection of rhMANF or rhCDNF on d7 after dMCAo. No differences were found in the number of neurons or CD68+ cells in the thalamus at d14 after dMCAo but the neurological deficits were milder in the rhMANF and rhCDNF groups compared to the vehicle group. The recovery-promoting mechanism of single post-stroke MANF and CDNF protein injection remains elusive but may be a result of enhanced local glia function or other effects that would facilitate neurotransmission.

6.4 INTRANASAL MANF DELIVERY

In an attempt to develop a non-invasive way to administer MANF for stroke treatment we used intranasal delivery of recombinant MANF in a neuroprotection paradigm. Intranasal delivery of other proteins, such as IGF-1, erythropoietin, and VEGF, has been shown to be a feasible way for neuroprotection in experimental ischemic stroke (Liu *et al.*, 2001; Grasso *et al.*, 2007; Buck *et al.*, 2008; Yang *et al.*, 2009) and for passing the BBB (Chen *et al.*, 1998; Thorne *et al.*, 2004; Alcala-Barraza *et al.*, 2010).

Either 20 μg or 60 μg of MANF divided into three doses protected from ischemic stroke and ischemia-induced neurological deficits. However, the amount of rhMANF found in the brain after intranasal delivery was very low and detectable only with radiolabeling of the protein, implying that rhMANF would be efficient in only picomolar concentrations in the brain. To verify that the detected cerebral radioactivity was indeed from ^{125}I-rhMANF and not from free ^{125}I after protein degradation, a more sensitive ELISA method would be needed. Also, the calculated bioavailability of ^{125}I-rhMANF in the blood (0.4%) was higher compared to the measured bioavailability of unlabeled rhMANF in the serum (0.05%) which may suggest either counting of free radioactivity, binding of rhMANF to a carrier protein masking the epitopes for ELISA, or binding of rhMANF to blood cells that are removed during sera sample preparation.

Since rhMANF was given before ischemia, we measured cortical blood flow during ischemia but found no difference compared to vehicle treatment,

confirming the neuroprotective effect was not due to improved blood flow. Most rhMANF after intranasal delivery was detected in the systemic circulation and could also exert neuroprotective effects *via* systemic mechanisms. Given that MANF has been shown to have immunomodulatory effects, MANF could mediate its beneficial effects by modulating peripheral leukocytes known to play a role in the acute phase of ischemic stroke. We did study whether intranasal rhMANF affects the extravasation of Evans blue albumin, thought to reflect the leakiness of the BBB, but found no difference between rhMANF and vehicle treatment, indicating that the neuroprotective mechanism after intranasal rhMANF delivery is not related to decreased BBB damage.

In this thesis, we conducted a proof-of-concept study showing that intranasal delivery of rhMANF has neuroprotective effects. We did not use any means to increase the nasal absorption, such as adsorption enhancers, and the bioavailability of rhMANF was, indeed, very low. Considering the potential clinical use of MANF in the future, it is clear that intracranial delivery is not a realistic option in routine stroke therapy. Therefore, other delivery routes should be considered and intranasal delivery is one potential option shown to work clinically with insulin (Reger *et al.*, 2008). However, there are drastic differences in the anatomy of nasal pathways and distances within the brain between rodents and humans (Lochhead & Thorne, 2012), making intranasal delivery far more inefficient in patients and viable only for molecules effective in very low concentrations. Another option could be to use the stroke-induced disruption of the BBB as a means to deliver BBB-impermeable drugs to the infarcted brain from systemic circulation. In the MCAo rat model, leakage of large molecules through the BBB has already been reported 25 min after reperfusion (Strbian *et al.*, 2008; Abo-Ramadan *et al.*, 2009) and in ischemic stroke patients at least between 6-48h after stroke onset (Merali *et al.*, 2017). However, rapid degradation of proteins in the systemic circulation and potential systemic adverse effects could pose problems in the systemic delivery approach, making intranasal delivery a more sophisticated technique in these aspects. Collectively, before intranasal rhMANF therapy could be considered as a clinical alternative, serious efforts for improving the bioavailability and developing an effective formulation would need to be made.

6.5 TRANSLATIONAL ASPECTS AND FUTURE PROSPECTS

Stroke Therapy Academic Industry Roundtable (STAIR) recommendations for conducting preclinical stroke studies were created after the massive failure of experimental compounds in clinical trials in the 1990s (Stroke Therapy Academic Industry, 1999). Lack of internal validity is common in preclinical stroke studies and small sample sizes, lack of blinding and randomization, and

publication bias have led to an overestimation of the efficacy of neuroprotective treatments (Macleod *et al.*, 2008; Kleikers *et al.*, 2015). Also, lack of comorbidity in animal models poses translational issues, and at least in the case of a free radical scavenger, the efficacy was significantly lower in hypertensive rats compared to normotensive (Macleod *et al.*, 2008). It is important to perform studies in a blinded and randomized manner in several species and in more than one laboratory, in both permanent and transient MCAo models, with more than one outcome measure in both acute and long-term phases (Stroke Therapy Academic Industry, 1999). Furthermore, sex differences and toxicology should be comprehensively studied before proceeding to clinical trials. STAIR encourages studying multiple pharmacological approaches simultaneously as targeting several different pathophysiological mechanisms may lead to a more favorable outcome than targeting only one mechanism. In this regard, MANF seems like a potential molecular probe for a drug candidate as it has several effects. However, the immune systems of laboratory mice have been shown to resemble more neonatal than adult human (Beura *et al.*, 2016) and housing animals in conditions that are too clean may pose translational issues especially when assessing the efficacy of immunomodulatory therapies.

While the STAIR recommendations focused mainly on stroke neuroprotection studies, specific guidelines were also created for preclinical stroke rehabilitation and recovery studies by the Stroke Recovery and Rehabilitation Roundtable (SRRR) (Corbett *et al.*, 2017). The SRRR recommendations focus on the importance of relevant long-term outcome measures during the months of stroke recovery monitoring, such as finding suitable tests for the detection of chronic impairment and implementation of brain imaging comparable to clinical practice.

With keeping these aspects in mind, the therapeutic effect of post-stroke MANF and naloxone treatment should be confirmed in female and old animals and in a thromboembolic model that better reflects the natural course of human infarcts, and preferably also in hypertensive animals to better depict the comorbidity in stroke patients. Also, long-term recovery studies should be performed using behavioral tests sensitive for detecting chronic impairment. Good brain penetration properties and decades of clinical experience make naloxone an interesting candidate with relatively newly uncovered anti-inflammatory effects and the potential of repeated naloxone dosing in ischemic stroke patients' treatment should be considered.

Also, more studies on the molecular targets of MANF should be conducted to reveal the mechanism behind MANF's recovery-promoting effects. Regarding the possible role of MANF in the regulation of immune cell function, it would be highly interesting to study whether the recovery from stroke is hindered in mice with myeloid cell-specific MANF deletion. Our post-stroke AAV-MANF data indicate that enhanced removal of tissue debris by

phagocytic cells may promote recovery, further contributing to the increasing amount of reports supporting pro-reparative functions of immune cells. Thus, future stroke therapies may focus on enhancing these reparative functions of immune cells rather than blocking the overall post-stroke inflammation that has long been the trend in experimental stroke research.

7 CONCLUSIONS

In this thesis work, we aimed to characterize post-stroke neuroinflammation in the dMCAo model, including the secondary pathology of the thalamus, and MANF protein expression pattern in infarcted human and rodent brain. We also investigated pharmacological post-stroke therapies, naloxone and MANF, that have been associated with immunomodulatory properties, and conducted a proof-of-concept study on non-invasive intranasal MANF therapy. Based on this work, the following conclusions can be made:

1) Post-stroke neuroinflammation is long-lasting and present for up to 4 months after dMCAo, especially in the ipsilateral thalamus. MANF protein expression is induced in activated myeloid cells and coexpresses with the phagocytic marker CD68 in the infarcted brain regions of patients, rats, and mice.

2) Secondary neuronal loss in the ipsilateral thalamus occurs between d7 and d14 post-stroke and is not associated with hyperalgesia. Intrathalamic injection of MANF or its homolog CDNF on post-stroke d7 has no effect on the neuronal loss or the number of CD68+ cells in the thalamus.

3) Post-stroke intranasal (+)-naloxone treatment continued for one week reduces behavioral deficits and decreases infarct size and microglia/macrophage activation.

4) Post-stroke AAV-MANF and rhMANF treatment promote functional recovery. Post-stroke AAV-MANF modulates inflammation by transiently increasing the number of phagocytic CD68+ cells and *C3* and *Emr1* transcripts in the peri-infarct area at d4 post-stroke.

5) Intranasal rhMANF treatment reduces infarct volume and leads to milder neurological deficits.

These results mirror the complexity and dual role of post-stroke inflammation as we found both the reduction of microglia/macrophage activation with (+)-naloxone and the increase of phagocytic cells with AAV-MANF promoted recovery in the dMCAo model. Post-stroke inflammation is a delicate balance between pro-inflammatory and pro-regenerative mechanisms where the timing of therapy is essential and both dampening the pro-inflammatory facet or boosting the pro-regenerative side can lead to a favorable outcome.

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